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13. ABSTRACT (Maximum 200 Words)

Activation of tyrosine kinases plays a key role in cell proliferation, and ErbB receptor tyrosine kinases are specifically implicated in breast cancer. Biochemical studies have recently identified the proto-oncogene product Cbl as a negative regulator of EGF receptor and ErbB2. The Cbl-dependent negative regulation of ErbB receptors was associated with their ubiquitin modification and down-regulation from the cell surface. Based on these observations, this proposal is investigating the role of Cbl-mediated ubiquitination as a signal for targeting activated ErbB receptors to lysosomes where they undergo degradation. The work reported here has demonstrated that ubiquitin modification of EGFR is essential for its down-regulation. Furthermore, this modification is shown to be essential for EGFR trafficking between early and late endosome, while being dispensable for initial endocytosis. Further studies aim to establish the relative role of Cbl-dependent ubiqiuitinylation versus other endocytic motifs in ErbB receptor internalization, and dissect out components of the biochemical machinery that mediates ubiquitin-dependent lysosomal sorting of ErbB receptors. Elucidation of this novel pathway of ErbB receptor downregulation is likely to reveal novel targets to develop rational therapeutic agents for breast cancers with aberrant expression and/or activity of ErbB receptors.

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A Novel Pathway to Down-Regulate ErbB Signaling in Mammary Epithelial Cells

Introduction:

The experiments proposed in this application are designed to test a unique hypothesis that the proto-oncogene product Cbl down-regulates ErbB receptors by controlling their ligand-induced ubiquitination, internalization and degradation. Genetic studies, initially in C. elegans and Drosophila systems and recently using mouse knock-outs, as well as extensive biochemical studies have established Cbl as a negative regulator of tyrosine kinases. At the time of this application, the TKB domain of Cbl had been shown to be crucial for Cbl function, which formed the basis for the use of TKB domain mutants in our studies. Subsequent studies in our laboratory demonstrated that the RING finger domain of Cbl was crucial for negative regulation of EGFR and Syk tyrosine kinases. Furthermore, the TKB and RING finger domain (together with short conserved regions surrounding the RING finger domain) were sufficient for Cbl-induced negative regulation of EGFR and Syk, as well as for Cbl-mediated ubiquitination of EGFR. Given the ability of Cbl to support the ubiquitination of target proteins in vitro, the TKB and RING finger domains of Cbl together define a novel ubiquitin ligase module that specifically targets activated tyrosine kinases like ErbB receptors for ubiquitination. Understanding the role of this novel biochemical machinery and how ubiquitin modification mediates the negative regulation of ErbB receptors is likely to provide crucial insights of biological and medical significance.

Body:

Studies carried out as a result of this trainee award helped further the training of the initially awarded trainee (Dr. Karen Mullane-Robinson) and the fellow that replaced her on the project when she moved to a biotechnology position (Dr. Lei Duan). The studies carried out during the funding period have helped demonstrate that Cbl-dependent ubiquitin modification of EGF receptor is indispensable for its lysosomal sorting but dispensable for internalization. Based on these novel insights, new EGFR mutants have been engineered to further clarify the possible redundant role of Cbl and ubiquitin machinery in EGFR internalization and to investigate the biochemical machinery that is responsible for Cbl and ubiquitin-dependent sorting of EGFR to lysosomes. Additional studies have helped uncover the role of Cbl as a negative regulator of downstream components of ErbB receptor signaling.

Ligand-induced EGFR ubiquitinylation and degradation are impaired in cells lacking endogenous Cbl expression: Previous studies supporting a role for Cbl in EGFR ubiquitinylation and downregulation have exclusively used overexpression of wildtype Cbl or its dominant negative mutants (Ref. 17, 18, 42 in Duan et al.). However, the role of endogenous Cbl in EGFR ubiquitinylation and degradation has not been addressed directly. To address this question, we derived two distinct pairs of Cbl+/+ and

Cbl-/- mouse embryo fibroblast cell lines (MEFs), using Cbl-/- mouse lines developed independently in the Bowtell and Gu laboratories (34, 35 in Duan et al.); these cell lines are designated Cbl+/+ (DB) and Cbl-/- (DB), and Cbl+/+ (HG) and Cbl-/- (HG), respectively. Western blotting of whole cell lysates confirmed the Cbl protein expression in the Cbl+/+ MEFs but not in either of the Cbl-/- MEFs (Fig. 1A in Duan et al.). The Cbl-/-(DB) MEFs express low levels of a truncated Cbl protein (data not shown) representing a nonfunctional splice product, as previously reported (34, 37 in Duan et al.). Since other Cbl family members, such as Cbl-b and Cbl-c, may also play a role in EGFR downregulation (43, 44 in Duan et al.), we wished to determine the level of expression of Cbl-b and Cbl-c in these MEFs. To assess the expression of Cbl-b, we used an antibody (H454) that recognizes both Cbl and Cbl-b (determined using HA-tagged Cbl and Cbl-b proteins; data not shown). We found that Cbl-b protein was detectable in the lysates of Cbl+/+ and Cbl-/- MEFs from which all Cbl protein had been immunodepleted using a Cbl-specific antibody (Fig. 1B in Duan et al., lanes 5-8); notably, the level of Cbl-b protein was substantially lower in the Cbl-/- (HG) MEFs. We also assayed for the presence of Cbl-c by Northern blot, as no antibody is currently available; the Cbl-c mRNA levels were nearly undetectable in all MEFs and there was no compensatory increase in Cbl-/- cells (data not shown). While the reasons for reduced Cbl-b expression in the Cbl-/- (HG) MEFs are unknown, this trait was advantageous to assess the contribution of endogenous Cbl to EGFR downregulation. To analyze the impact of Cbl deficiency on EGFR downregulation, we used retroviral infection to derive stable transfectants of the Cbl+/+ and Cbl-/- MEFs expressing human EGFR on the cell surface (Fig. 1C and 1D in Duan et al.).

As Cbl is an EGFR-directed ubiquitin ligase (10-12), we first assessed the ligand-induced EGFR ubiquitinylation in EGFR-expressing Cbl+/+ and Cbl-/- MEFs. Serum-starved cells were stimulated with EGF for various times, and anti-EGFR immunoprecipitates (IPs) were subjected to immunoblotting (IB) with an anti-ubiquitin antibody. As anticipated, ligand-dependent ubiquitinylation of EGFR was readily detected in both Cbl+/+ MEF lines (DB and HG; Fig. 2A in Duan et al.). Notably, ligand-induced EGFR ubiquitinylation in both Cbl-/- MEF lines was greatly reduced, indicating that endogenous Cbl is the major ubiquitin ligase involved in EGFR ubiquitinylation in MEFs. Importantly, anti-EGFR blotting of anti-EGFR IPs (Fig. 2A in Duan et al., second panel) or whole cell lysates (Fig. 2B in Duan et al.) revealed that the ligand-induced loss of EGFR protein was substantially slower in both Cbl-/- MEFs; concomitantly, we observed a slower and less pronounced loss of phosphotyrosine (pY) signals on EGFR (Fig. 2A in Duan et al., bottom panel). The pattern of EGFR phosphorylation also indicates that impaired EGFR ubiquitinylation in Cbl-/- cells is not due to defective EGFR activation. To establish that defective EGFR ubiquitinylation and degradation in Cbl-/- MEFs was due to the lack of Cbl expression, and not an artifact of cell line derivation, we reconstituted Cbl expression in the EGFR-expressing Cbl-/- (HG) MEFs. IB of whole cell lysates demonstrated that retrovirus-mediated introduction of HA-tagged Cbl led to reconstitution of Cbl expression in Cbl-/- (HG) MEFs (Fig. 2C in Duan et al.).

Comparison of the HA-Cbl-transfected versus the vector-transfected cells demonstrated that reconstitution with Cbl fully restored the EGF-induced ubiquitinylation

and degradation of EGFR (Fig. 2D and 2E in Duan et al.). Thus, the defect in EGFR ubiquitinylation and degradation in Cbl-/- (HG) MEFs is solely due to lack of Cbl expression. Overall, the results with Cbl-/- MEFs and their Cbl-reconstituted derivatives establish a clear role for endogenous Cbl in ligand-induced EGFR ubiquitinylation and degradation.

EGFR internalization is unaltered in Cbl-/- MEFs: The precise site(s) of Cbl action in the endocytic trafficking of EGFR has not been clarified. Based on the formation of a Cbl-CIN85-endophilin complex, a recent set of studies concluded that Cbl plays an important role in the initial internalization of EGFR (25, 45 in Duan et al.). In addition, an EGFR mutant unable to bind Grb2 was impaired in its ability to form an EGFR-Grb2-Cbl complex, and to undergo ligand-induced ubiquitinylation, and internalization, further suggesting a role for Cbl-mediated ubiquitinylation in EGFR internalization (26 in Duan et al.). However, whether the effects of the CIN85 mutant are due to the loss of a Cbl-CIN85 interaction or of other protein-protein interactions, or whether Cbl-EGFR interaction through Grb2 is required for EGFR internalization, have not been established. In fact, the N-terminal half of Cbl, which included only the TKB and RING finger domains and lacked both the CIN85 and Grb2 interaction sites, was sufficient to enhance the ubiquitinylation and degradation of EGFR (17, 46, and 47 in Duan et al.). Moreover, overexpression of Cbl did not enhance the EGFR internalization (42 in Duan et al.), and dominant-negative Cbl mutants blocked ubiquitinylation and degradation but not the internalization of EGFR (17, 18 in Duan et al.). These discrepancies suggest that the impairment of EGFR internalization by the dominant interfering forms of CIN85 may not be through an interruption of the Cbl-CIN85 interaction but through another mechanism. Similarly, Cbl-Grb2 interaction may not be the basis for Grb2-regulated EGFR internalization, as Grb2 can also recruit a number of other proteins, such as RN-tre, to activated EGFR (48 in Duan et al.). To clarify the role of Cbl in EGFR internalization, we used the MEF system characterized above to directly establish if the loss of Cbl function had an obvious impact on ligand-induced internalization of EGFR. For this purpose, we assayed the acid-stable uptake of Alexa-Fluor-488-conjugated-EGF by MEFs.

To minimize the contribution of recycling and/or lysosomal degradation of internalized EGFR, we quantified the Alexa-488-EGF uptake in MEFs for relatively brief time periods (up to 10 min). We also used EGF at a relatively low concentration (25 ng/ml) that only saturates 40-50% of cell surface EGFR in our system (data not shown). The rate of initial EGFR internalization in Cbl-/- (HG) MEFs was comparable to that in Cbl+/+ (HG) MEFs (Fig. 3A in Duan et al., $P>0.05$). Furthermore, the rate of initial internalization of EGFR in Cbl-reconstituted and vector-transfected Cbl-/- (HG) MEFs was essentially identical (Fig. 3C in Duan et al., left panel, $P>0.05$), even though the downregulation of EGFR at later time points, which reflects a net balance of internalization, recycling and lysosomal degradation, was dramatically slower in Cbl-/- (HG) MEFs compared to Cbl-reconstituted MEFs (Fig. 3C in Duan et al., right panel, $P<0.001$). Unimpaired EGFR internalization in Cbl-/- (HG) MEFs was further confirmed by the immunofluorescence staining of EGFR after EGF stimulation. The pattern of EGFR-staining endocytic vesicles 10 min after EGF stimulation was comparable in Cbl-

/- and Cbl+/+ (HG) MEFs (Fig. 3B in Duan et al.). The combination of unimpaired initial internalization and reduced EGFR degradation (and downregulation) in Cbl-/- MEFs indicates that Cbl function is critical at a post-internalization step in EGFR downregulation but not at the internalization step.

CHO-Ts20 cells demonstrate that the ubiquitin pathway is essential for EGFR degradation but dispensable for internalization: Previous analyses using overexpression of wildtype or mutant forms of Cbl have demonstrated a tight correlation between the Cbl-regulated ubiquitinylation and degradation of EGFR (49 in Duan et al.). This correlation is further strengthened by studies of EGFR mutants unable to bind to Cbl (50 in Duan et al.), and analyses of Cbl-deficient MEFs presented above. However, the questions of whether Cbl-mediated EGFR degradation proceeds through ubiquitinylation and whether the ubiquitin pathway is required to target EGFR for lysosomal degradation have not been directly addressed. To clarify these issues, we utilized a CHO cell line with a temperature-sensitive ubiquitin-activating (E1) enzyme, which results in a conditional defect in protein ubiquitinylation (51 in Duan et al.).

As CHO cells lack EGFR, the CHO cell line with mutant E1 (CHO-Ts20) and its wildtype counterpart (CHO-E36) were stably transfected with human EGFR. Transient overexpression of wildtype Cbl, in comparison with vector control, enhanced the ubiquitinylation of EGFR in Ts20-EGFR cells (Fig. 4A in Duan et al., compare lanes 2 and 4). In contrast, the overexpression of a Cbl RING finger domain mutant, Cbl-C3AHN, suppressed the ligand-induced ubiquitinylation (Fig. 4A in Duan et al., lane 6) and degradation of EGFR (Fig. 4B in Duan et al., lanes 7-9). Thus, both ubiquitinylation and degradation of EGFR in CHO-Ts20 cells are dependent on endogenous Cbl.

To directly assess the impact of inhibiting ubiquitinylation in CHO-Ts20 cells on EGFR degradation, we compared Ts20-EGFR and E36-EGFR (control) cell lines at 30°C, the permissive temperature for ubiquitinylation, versus 42°C, the non-permissive temperature. When assayed at 30°C, the ligand-induced EGFR ubiquitinylation was observed in both Ts20-EGFR and E36-EGFR cells (Fig. 5A and 5B in Duan et al., lanes 1-3 and 7-9). In contrast, ligand-induced EGFR ubiquitinylation was markedly attenuated in Ts20-EGFR cells shifted to 42°C (Fig. 5A in Duan et al., lanes 10-12); as a control, the ubiquitinylation of EGFR in E36-EGFR cells was unaffected by the temperature shift (Fig. 5A in Duan et al., lanes 4-6). Analysis of EGFR protein levels over a longer time course of EGF stimulation demonstrated that the kinetics of EGFR degradation in E36-EGFR cells was similar at 30°C versus 42°C (Fig. 5B in Duan et al., compare lanes 1-4 and 5-8). In contrast, the degradation of EGFR in Ts20-EGFR cells was retarded when these cells were shifted to 42oC to block ubiquitinylation (Fig. 5B in Duan et al., compare lanes 9-12 and 13-16). The requirement of Cbl for EGFR degradation, together with the inhibition of EGFR degradation upon blockade of the ubiquitinylation pathway in Ts20-EGFR cells, supports the view that it is the ubiquitin ligase activity of Cbl rather than another function that is essential in Cbl-mediated EGFR degradation. Interestingly, in both Cbl-/- MEF cell lines that we utilized, we observed a low level of residual EGFR ubiquitinylation upon ligand stimulation. Whether this is due to a low level of Cbl-b expression or due to an unrelated ubiquitin ligase capable of targeting EGFR for ligand-

induced ubiquitinylation remains to be investigated. The residual ubiquitinylation could account for the EGFR degradation that is still observed. Alternatively, the continued EGFR degradation could reflect the contribution of other mechanisms, such as those mediated by the dileucine motifs, to endosomal sorting of EGFR (52 in Duan et al.). Our finding that the initial internalization of EGFR was intact in Cbl-/- MEFs suggested that Cbl and Cbl-mediated EGFR ubiquitinylation was dispensable for EGFR internalization, but left open the possibility that ubiquitinylation of another protein(s) may mediate the internalization. In this regard, EGF-inducible ubiquitinylation of endocytic proteins, such as Eps15 (53 in Duan et al.) and CIN85 (54 in Duan et al.), has been previously demonstrated, although the role of the ubiquitinylation in EGFR internalization remains to be established. Ubiquitin has been clearly recognized as a receptor internalization motif in yeast (20, 21 in Duan et al.), while studies of the growth hormone receptor in mammalian cells indicate that ubiquitinylation of an unknown non-receptor component may be crucial for internalization (22 in Duan et al.). The Ts20-EGFR system provided a suitable system to test if the ubiquitin pathway was also essential for initial internalization of EGFR. Therefore, we also examined the EGFR internalization in Ts20-EGFR and E36-EGFR cells, using the assay described above. Similar to control E36-EGFR cells (Fig. 6 in Duan et al., left panel, $P>0.05$), the rate of initial EGFR internalization in Ts20-EGFR cells grown at 30°C and 42°C was comparable (Fig. 6B in Duan et al., right panel, $P>0.05$). Thus, the ubiquitinylation machinery is dispensable for the initial internalization step in the endocytic traffic of EGFR. Our results indicate that neither EGFR ubiquitinylation nor the monoubiquitinylation of other proteins, such as Eps15 and CIN85, is required for EGFR internalization. Dikic and colleagues have, however, observed that a dominant negative mutant of CIN85, which is not monoubiquitinylated by Cbl upon EGF stimulation, impairs the internalization and degradation of EGFR (23, 45 in Duan et al.). It is likely that the dominant negative form of CIN85 fails to interact with other partners, which may relate to its role at the internalization step. Furthermore, Dikic and colleagues observed that the Cbl-dependent monoubiquitinylation of CIN85 primarily occurred after EGFR endocytosis (23 in Duan et al.), which suggests a role for ubiquitinylated CIN85 at a post-endocytic step, consistent with our conclusions. Our results do not exclude the possibility that ubiquitinylation can function as an endocytic signal. In fact, monoubiquitinylation of EGFR is an enough signal for EGFR internalization (55 in Duan et al.). Our results do indicate, though, that this cannot be the sole mechanism for initial internalization, and that other mechanisms can fully support the internalization process in the absence of the ubiquitinylation of EGFR and any accessory proteins. The molecular nature of these additional signals remains to be fully elucidated. Several endocytic motifs have been identified in EGFR, including the dileucine-based motif (56 in Duan et al.) and the tyrosine-based AP-2 binding motif (57 in Duan et al.). However, mutational analyses suggest that none of these motifs is essential for internalization (56, 57 in Duan et al.). It is likely that a complex RTK such as EGFR has evolved multiple redundant mechanisms to ensure internalization, as this is a key regulatory process.

The internally controlled Ts20-EGFR and E36-EGFR cell pair also provided an opportunity to assess the nature of the endocytic compartment(s) where Cbl functions and

where ubiquitinylation plays a decisive role in the endocytic trafficking of internalized EGFR. For this purpose, we assessed the colocalization of the internalized EGFR with selected endocytic markers, using confocal microscopy. In both E36-EGFR and Ts20-EGFR cells grown at 30°C, the EGFR-containing vesicles were peripherally distributed at 5 min after EGF stimulation (Fig. 7A in Duan et al., B1 and E1) and gradually moved near the center of the cells, forming larger clusters by 30 min (Fig. 7A in Duan et al., C1 and F1). When E36-EGFR and Ts20-EGFR cells were compared at 42°C, the EGFR staining pattern at 5 min of EGF stimulation was similar (Fig. 7B, compare B1 and E1), consistent with the unaltered EGFR internalization seen in the internalization assay. Notably, however, the distribution of EGFR containing vesicles in Ts20-EGFR versus E36-EGFR cells grown at 42°C and stimulated for 30 min was quite distinct; these vesicles failed to move near the center and remained near the periphery in Ts20-EGFR cells (Fig. 7B in Duan et al., compare C1 and F1).

To characterize the EGFR-containing endosomal compartments, we either loaded the cells with fluorescent transferrin to mark the early/recycling endosomes, or carried out double staining for EGFR (Red) and LAMP-1 (36), a late endosome/lysosome marker; the cells were then analyzed using confocal microscopy. At 5 min of EGF stimulation at 30°C, the internalized EGFR in peripherally distributed vesicles mostly colocalized with transferrin (Fig. 7A in Duan et al., B3 and B4; E3 and E4; yellow); this colocalization was lost by 30 min of stimulation, when the EGFR was predominantly centrally clustered (Fig. 7A in Duan et al., C3 and C4; F3 and F4; red). However, EGFR in these vesicles colocalized with LAMP-1 (Fig. 8 in Duan et al., B3 and B4; E3 and E4; yellow), indicating that a proportion of internalized EGFR underwent a time-dependent endocytic transport from early to late endosomes. This pattern remained unaltered when the E36-EGFR control cells were examined at 42°C (Fig. 7B in Duan et al., C3 and C4). In contrast, when Ts20-EGFR cells were grown at 42°C to disrupt ubiquitinylation, the internalized EGFR remained colocalized with transferrin at 30 min of EGF stimulation (Fig. 7B in Duan et al., F3 and F4; yellow), and showed a lower degree of colocalization with LAMP-1 (Fig. 8 in Duan et al., F3 and F4). These results indicate that ubiquitinylation is crucial for an early endosome to late endosome sorting step in the endocytic trafficking of ligand-stimulated EGFR. The simplest interpretation of these results is that Cbl-mediated ubiquitinylation of EGFR (and/or other accessory proteins) serves as an endosomal sorting signal for EGFR delivery from early to late endosome/lysosome, and that ubiquitin modification serves as an essential signal at this step to ensure the efficient delivery of EGFR into the degradative compartments of the endocytic machinery. This model is consistent with the previous finding that ubiquitinylation and proteasome activity is needed for EGFR transfer into internal vesicle of MVBs (28 in Duan et al.). It is also compatible with the genetically defined role of ubiquitinylation of yeast transmembrane receptors for sorting into the inner vesicles of the MVB (58, 59 in Duan et al.). In fact, yeast studies have identified endosomal sorting of receptor traffic (ESCRTs) complexes, such as ESCRT-1 (60 in Duan et al.), that function as ubiquitin recognizing proteins within the endocytic system. Deletion of TSG-101, one of the ESCRT-1 complex components in mammals, retards EGFR degradation and causes accumulation of ubiquitinylated proteins on endosomes (61 in Duan et al.). Furthermore, the hepatocyte growth factor receptor substrate (Hrs), the mammalian

counterpart of the yeast ESCRT-2 protein also has a ubiquitin interacting motif (UIM) (53, 62, and 63 in Duan et al.). A truncated Hrs protein lacking the UIM impaired EGFR degradation in *Drosophila*, by affecting endosome membrane invagination and MVB formation (64 in Duan et al.). Thus, Cbl-mediated ubiquitinylation could represent the receptor modification required for recognition by mammalian ESCRT proteins to facilitate endosomal sorting of EGFR to lysosomes. Whether or not the ubiquitin modification provides the signal for delivering EGFR into the inner vesicles of the MVB in mammalian cells, and the regulatory control of this process, is an important area of future investigation. In conclusion, our studies utilizing two distinct and independent experimental systems provide strong evidence that Cbl-mediated ubiquitinylation is essential for efficient ligand-induced degradation of EGFR, but is dispensable for initial receptor internalization. Future studies should address whether this dichotomy is a general feature of Cbl regulation of RTKs and other transmembrane receptors.

Generation of EGFR mutants disabled in Cbl binding, endocytic motifs or both: Given our dramatic observations that Cbl-mediated ubiquitinylation is dispensable for EGFR internalization, we have now set out to dissect out if Cbl has any role (redundant or non-redundant) in EGFR internalization. For this purpose, we have engineered the following EGFR mutants: EGFR-Y1045F (Cbl TKB domain binding mutant; Waterman H, et al. EMBO J. 21:303-313, 2002); EGFR-Y1068F and EGFR-Y1086F (defective in indirect Cbl binding via Grb2; Fukazawa T et al. J Biol. Chem. 271:14554-14559, 1996; Waterman H, et al. EMBO J. 21:303-313, 2002); RGFR-L672A, EGFR-L674A, EGFR-L672A/L674A (defective in AP2 binding site and hence lacking an internalization motif; and EGFR-Y1045F/Y1068F/L672F/L674F (lacking Cbl binding and AP2 binding motif). These mutants are being cloned in pMSCV-puro vector for expression in Cbl+/+ and Cbl-/- MEFs to discern the role of Cbl versus AP2 pathway in EGFR internalization.

Cbl-mediated ubiquitinylation and negative regulation of Vav: In an extension of studies on the regulation of EGFR by Cbl-dependent ubiquitinylation, we have recently initiated studies on the potential role of Cbl to control downstream mediators of EGFR signaling. These studies have focused on Vav family of Rac small G-protein guanine nucleotide exchange factors (GEFs), which are known proto-oncogene products and play a role in signaling downstream of EGFR as well as other tyrosine kinase-coupled receptors. Vav is known to associate with Cbl ubiquitin ligase, but the consequences of this interaction remain to be elucidated. Using immortalized cell lines from $Cbl^{+/+}$ and $Cbl^{-/-}$ mice, and transfection analyses in 293T cells, we have now demonstrated that Vav undergoes Cbl-dependent ubiquitinylation under conditions that promote Cbl and Vav phosphorylation. Interaction with Cbl also induced the loss of phosphorylated Vav. In addition, activated Vav mutant (Vav-Y174F) was more sensitive to Cbl-dependent ubiquitinylation. We demonstrated that the Cbl-dependent ubiquitinylation of Vav required Cbl/Vav association through phosphorylated Y700 on Cbl, and also required an intact Cbl RING finger domain. Finally, using transfection analyses, we showed that Cbl, but not its ubiquitin ligase mutant, can inhibit Vav-dependent signaling. Thus, our findings strongly support the role of Cbl, via its ubiquitin ligase activity, as a negative regulator of activated Vav. Thus, Cbl can negatively

regulate receptor signals by direct ubiquitinylation of receptors as well as indirectly by targeting downstream mediators of their signals. Further studies on Cbl regulation of Vav function in the context of EGFR will shed important light on the physiological role of this regulatory mechanisms (see Miura et al. attached).

Key Research Accomplishments:

- Demonstrated that Cbl-dependent EGFR ubiquitination is essential for sorting EGFR at a post-endocytic step but is dispensable for internalization
- Generated a panel of new EGFR mutants carrying mutations in the Cbl docking sites as well as conventional internalization motifs.
- Demonstrated that Cbl-dependent ubiquitinylation provides negative regulation of Vav, a downstream mediator EGFR signals.
- Generated a panel of GFP-tagged Cbl mutants.
- Generated Cbl+/+ and Cbl-/- MEFs and their EGFR and ErbB2 transfectants.
- Generated stable EGFR transfectants of wildtype and mutant CHO cells carrying a temperature-sensitive mutation in the ubiquitin attachment machinery.
- Generated a yellow fluorescent protein-tagged ubiquitin (YFP-Ub) expression plasmid.
- Demonstrated an essential role for EGFR ubiquitination in its degradation, using a genetic approach.
- Demonstrated that EGFR ubiquitination is not required for initial internalization
- Demonstrated that lack of ubiquitination of EGFR results in its failure to traverse from early to late endosome.
- Demonstrated that Cbl-dependent EGFR ubiquitination involves predominantly mono- and di-ubiquitin modifications.

Reportable Outcomes:

Publications:

1. Duan L, Miura Y, Dimri M, Majumder B, Dodge IL, Lakku Reddi A, Ghosh AK, Fernandes N, Zhou P, Mullane-Robinson K, Rao N, Donoghue S, Rogers RA, Bowtell D, Naramura M, Gu H, Band V, Band H. Cbl-mediated ubiquitinylation is required for lysosomal sorting of EGF receptor but is dispensable for endocytosis. **J. Biol. Chem.** 2003 May 18 [Epub ahead of print].

2. Miura-Shimura Y#, Duan L#, Rao NL, Lakku Reddi A, Shimura H, Rottapel R, Druker BJ, Tsygankov A, Band V, Band H.(#contributed equally) Cbl-mediated ubiquitinylation and negative regulation of Vav. **Submitted**.

3. Duan L, Majumder B, Mullane-Robinson K, Band H. EGF receptor down-regulation through Cbl-mediated ubiquitination. Poster presentation at the Era of Hope Meeting, Department of Defense Breast Cancer Research Program. Orange County Convention Center, Orlando, Florida. September 25-28, 2002 (Abstract P21-5).

4. Duan L, Miura Y, Dimri M, Majumder B, Dodge I, Lakkureddi A, Ghosh A, Fernandes N, Zhou P, Mullane-Robinson K, Rao N, Donoghue S, Bowtell D, Gu H, Naramura M, Band V, Band H. Cbl-mediated ubiquitination is required for lysosomal sorting of EGF receptor but is dispensable for endocytosis. Poster presentation at the 2002 Research fellows' Poster Session, Brigham and Women's Hospital, Boston.

5. Duan L, Miura Y, Band H. Cbl-mediated ubiquitination is required for lysosomal sorting of EGF receptor but is dispensable for endocytosis. Poster and oral presentation at the FASEB Experimental Biology conference, New Orleans, April 2003 (abstract #629.6).

Reagents:

- Generated a panel of new EGFR mutants carrying mutations in Cbl docking sites as well as conventional internalization motifs

- Generated wildtype and mutant constructs of Vav

- Generated Cbl mutants deficient in ubiquitin ligase function, EGFR interaction and Vav interaction

- Generated a panel of GFP-tagged Cbl mutants.

- Generated Cbl+/+ and Cbl-/- MEFs and their EGFR and ErbB2 transfectants.

- Generated stable EGFR transfectants of wildtype and mutant CHO cells carrying a temperature-sensitive mutation in the ubiquitin attachment machinery.

- Generated a yellow fluorescent protein-tagged ubiquitin (YFP-Ub) expression plasmid.

Funding applied for based on this work:

The work carried out under this award is part of the background and preliminary studies for two NIH RO1 applications (Molecular Control of EGF Receptor Down-Regulation; PDGF Receptor Regulation by Cbl) that were submitted by Dr. Band, and have now received funding. Thus, the studies initiated through this training award will be fully followed up by the PI as a member of Dr. Band's laboratory.

Manuscripts included:

1. Duan L, Miura Y, Dimri M, Majumder B, Dodge IL, Lakku Reddi A, Ghosh AK, Fernandes N, Zhou P, Mullane-Robinson K, Rao N, Donoghue S, Rogers RA, Bowtell D, Naramura M, Gu H, Band V, Band H. Cbl-mediated ubiquitinylation is required for lysosomal sorting of EGF receptor but is dispensable for endocytosis. **J. Biol. Chem.** 2003 May 18 [Epub ahead of print].

2. Miura-Shimura Y[#], Duan L[#], Rao NL, Lakku Reddi A, Shimura H, Rottapel R, Druker BJ, Tsygankov A, Band V, Band H.(#contributed equally) Cbl-mediated ubiquitinylation and negative regulation of Vav. Submitted

Conclusions:

In conclusion, we demonstrate that endogenous Cbl is essential for ligand-induced ubiquitinylation and efficient degradation of EGFR. Further analyses using CHO cells with a temperature-sensitive defect in ubiquitinylation confirm a crucial role of the ubiquitin machinery in Cbl-mediated EGFR degradation. However, internalization into early endosomes did not require Cbl function or an intact ubiquitin pathway. Confocal immunolocalization studies indicated that Cbl-dependent ubiquitinylation plays a critical role at the early endosome to late endosome/lysosome sorting step of EGFR downregulation. These findings establish Cbl as the major endogenous ubiquitin ligase that is responsible for EGFR degradation, and show that the critical role of Cbl-mediated ubiquitinylation is at the level of endosomal sorting, rather than at the level of internalization. These findings provide critical new insights into our understanding of ErbB receptor downregulation. Further studies have identified the downstream signaling protein Vav, a Rac GEF, as a target of Cbl-mediated ubiquitinylation, thus revealing an additional biochemical mechanism for Cbl-dependent downregulation of ErbB receptor signaling.

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Cbl-mediated ubiquitinylation is required for lysosomal sorting of EGF receptor but is dispensable for endocytosis

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Running Title: Cbl is required for EGFR degradation but not for internalization

Abstract:

Ligand-induced downregulation controls the signaling potency of the EGF receptor (EGFR/ErbB1). Overexpression studies have identified Cbl-mediated ubiquitinylation of EGFR as a mechanism of ligand-induced EGFR downregulation. However, the role of endogenous Cbl in EGFR downregulation and the precise step in the endocytic pathway regulated by Cbl remain unclear. Using $Cbl^{-/-}$ mouse embryonic fibroblast (MEF) cell lines, we demonstrate that endogenous Cbl is essential for ligand-induced ubiquitinylation and efficient degradation of EGFR. Further analyses using CHO cells with a temperature-sensitive defect in ubiquitinylation confirm a crucial role of the ubiquitin machinery in Cbl-mediated EGFR degradation. However, internalization into early endosomes did not require Cbl function or an intact ubiquitin pathway. Confocal immunolocalization studies indicated that Cbl-dependent ubiquitinylation plays a critical role at the early endosome to late endosome/lysosome sorting step of EGFR downregulation. These findings establish Cbl as the major endogenous ubiquitin ligase responsible for EGFR degradation, and show that the critical role of Cbl-mediated ubiquitinylation is at the level of endosomal sorting, rather than at the level of internalization.

Introduction:

Growth factor receptor tyrosine kinases (RTKs) play crucial roles in cellular proliferation, survival, migration and differentiation. EGF receptor (EGFR/ErbB1) is a member of the ErbB family (ErbB1-4) of RTKs, which play crucial homeostatic roles and are implicated in oncogenesis. Ligand-induced activation of RTKs leads to the assembly of signaling protein complexes and subsequent activation of downstream signaling pathways. The ligand-activated RTKs also undergo rapid endocytosis (1). The endocytosed receptors then undergo a sorting process, which determines receptor fate and signal intensity. The receptors can be targeted to the lysosome for degradation, which terminates receptor signals. Alternatively, the internalized receptors can be recycled back to the cell surface for continued ligand binding and signaling (2-5). The relative efficiency of lysosomal sorting versus recycling is a key determinant of the signaling potency of RTKs (6). For example, EGFR is predominantly delivered to lysosomes when activated by EGF. In contrast, heregulin-activated ErbB2 is primarily recycled. The greater efficiency of the recycling process is thought to be a major determinant of the signaling superiority of ErbB2 over EGFR (7-9).

Despite a critical role of endocytic sorting as a determinant of ErbB receptor downregulation, the biochemical mechanisms that regulate this process have only recently begun to be elucidated. We, and others, have identified Cbl as one such regulator (10-12). Cbl is recruited to the activated EGFR through both direct and indirect binding. Direct Cbl-EGFR interaction is mediated through the N-terminal tyrosine kinase-binding (TKB) domain of Cbl, which binds to phosphorylated Y1045 on EGFR (13). Indirect Cbl-EGFR interaction is primarily mediated through Grb2: the SH3

domain of Grb2 binds to proline-rich sequences in Cbl whereas the SH2 domain binds to autophosphorylated EGFR (14). The RING finger domain in Cbl binds to ubiquitin-conjugating enzymes (E2s), allowing Cbl to function as a ubiquitin ligase (E3) towards activated EGFR (15,16). Overexpression studies have demonstrated that Cbl-mediated ubiquitinylation promotes downregulation of EGFR, whereas Cbl proteins with mutations in the RING finger domain, or in a conserved helix connecting the RING finger and TKB domains, reduce the extent of EGFR ubiquitinylation and downregulation (17-19). It has not been established, however, whether endogenous Cbl-mediated ubiquitinylation plays an essential role in EGFR degradation.

Two potential mechanisms for Cbl-mediated, and ubiquitinylation-dependent, EGFR downregulation have been proposed. First, a role for Cbl-mediated ubiquitinylation in EGFR endocytosis has been postulated based on the well-established role of ubiquitinylation in the endocytosis of yeast pheromone receptors (20,21), and the apparent requirement for the ubiquitin pathway in mammalian growth hormone receptor internalization (22). Consistent with this possibility, Cbl was also recently found to associate with a CIN85-endophilin complex and to facilitate the monoubiquitinylation of CIN85 (23). Since a truncated CIN85 mutant that did not associate with Cbl impaired the rate of EGFR internalization, the investigators concluded that Cbl played a role in EGFR endocytosis by serving as an adaptor to link EGFR with CIN85 and endophilin (24). Another study arrived at a similar conclusion in the context of the RTK c-MET (25). More recently, the EGFR Y1068/1086F mutant, which is unable to bind Grb2, was also found to be impaired for endocytosis (26). Interestingly, this mutant was also unable to efficiently recruit Cbl to EGFR, and showed impaired ubiquitinylation. Collectively,

these studies suggest a role for Cbl, and Cbl-regulated ubiquitinylation, in EGFR endocytosis. Other studies, however, argue against a role for Cbl-mediated ubiquitinylation in the initial internalization of EGFR. For example, even though EGFR ubiquitinylation occurs at the cell surface, it did not appear to be required for endocytosis (27,28). In contrast, both ubiquitinylation and proteasome activity were needed for transferring EGFR into internal vesicles of the multi-vesicular body (28). In other studies, dominant-negative Cbl mutants were found to prevent downregulation of EGFR, but EGFR was seen to undergo ligand-induced localization to intracellular vesicular structures (17). Furthermore, the rate of initial internalization did not correlate with the ability of overexpressed Cbl mutants to inhibit EGFR downregulation (18). These results support a role for Cbl and ubiquitinylation at a late step in the endocytic pathway, rather than at the initial internalization step. The relative contribution of these alternate mechanisms in Cbl-mediated downregulation of EGFR has not been clarified.

In the present study, we used CHO cells conditionally defective in ubiquitinylation, and Cbl-deficient mouse embryonic fibroblast (MEF) cells, to address the role of Cbl and Cbl-mediated ubiquitinylation in the internalization, endosomal sorting and degradation of EGFR. We demonstrate that Cbl-mediated EGFR ubiquitinylation is required for efficient sorting of activated EGFR into the lysosome for its degradation. In contrast, neither Cbl nor Cbl-mediated ubiquitinylation are required for initial EGFR endocytosis.

Material and Methods

Reagents:

Biotin-EGF complexed with Alexa-Fluor-488-labeled streptavidin, and Alexa-Fluor-488-conjugated transferrin were obtained from Molecular Probes Inc. (www.molecularprobes.com). Opti-MEM I Reduced Serum Medium was from Invitrogen Corporation (www.invitrogen.com). The Fugene-6 reagent was obtained from Roche Molecular Biochemicals (www.roche.com).

Antibodies:

The antibodies used in this study were: mouse anti-EGFR monoclonal (mAb) 528 (IgG2a) from ATCC; rat anti-LAMP-1 mAb and rabbit polyclonal (pAb) anti-EGFR antibody (sc-03) from Santa Cruz Biotechnology, Inc. (www.scbt.com); anti-ubiquitin mAb P4G7 (IgG1) from Covance Research Products Inc. (www.covance.com); anti-phosphotyrosine mAb 4G10 (IgG2a) from Dr. Brian Druker (Oregon Health Sciences University, Portland, OR); and pAb anti-LAMP-1 (931B) (29) from Dr. Minoru Fukuda (The Burnham Institute, CA). Cy3-conjugated goat-anti-mouse IgG and Cy2-conjugated goat-anti-rabbit IgG secondary reagents were from Jackson Immuno-Research Laboratories Inc. (www.jacksonimmuno.com).

DNA constructs and mutants:

pAlterMAX-HA-Cbl and pAlterMAX-HA-Cbl-C3AHN (RING finger mutant) (30), and pJZenNeo-HA-Cbl retroviral expression construct (31) have been described

previously. The human EGFR cDNA insert from pAlterMAX-EGFR construct (17) was cloned into pcDNA3 to generate pcDNA3-EGFR, and into pMSCV-puro retroviral vector (www.clonetech.com) to generate pMSCV-puro-EGFR.

Cell lines:

The Chinese Hamster Ovary (CHO) cell line with a temperature-sensitive E1 ubiquitin-activating enzyme, CHO-Ts20 and its wildtype control cell line, CHO-E36 (32) (from Dr. Ger Strous, University Medical Center, Utrecht, The Netherlands) were grown in MEM-a medium supplemented with 10% fetal bovine serum, and penicillin/streptomycin (www.invitrogen.com). Mouse embryonic fibroblast (MEF) cells were derived using standard isolation methods (33) from day 13.5 $Cbl^{-/-}$ and littermate $Cbl^{+/+}$ embryos, from two separate $Cbl^{-/-}$ mouse backgrounds (34,35), followed by the 3T3 protocol from passage 3 to 25 (36). These cells were grown in MEM-a medium with 10% FBS, 2 mM glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 0.1 mM non-essential amino acids, and penicillin/streptomycin. The MEF cells used here are independent of another pair that we have reported previously (37,38).

Transfections:

Transient or stable transfectants of CHO-Ts20, CHO-E36 expressing human EGFR (or its mutants) were generated by Fugene-6-mediated transfection of pcDNA3-EGFR, according to the manufacturer's instructions. Transfectants were selected in media containing 0.5 mg/ml G418 (www.invitrogen.com) and clones were analyzed for EGFR expression by immunoprecipitation and immunoblotting (described below).

Stable transfectants of $Cbl^{+/+}$ and $Cbl^{-/-}$ MEFs expressing human EGFR were established by retroviral infection as described previously (39). Cells were selected in puromycin (www.sigma-aldrich.com; 2.5 μg/ml and bulk transfectants were analyzed for EGFR expression using fluorescence-activated cell sorter (FACS) analysis with mAb 528 and immunoblotting with sc-03 anti-EGFR Ab.

Transfections were carried out with the Fugene-6 reagent. The amounts of input DNA are indicated in the figure legends. Cells were harvested at 48h after the addition of DNA precipitates.

EGF stimulation and preparation of cell lysates:

For EGF stimulation, cells were placed in starvation medium (growth medium containing 0.5% FBS) for 4-6h and then incubated with purified murine EGF (Catalog #E-4127, www.sigma-aldrich.com) for various lengths of time and at concentrations indicated in figure legends. Cells were rinsed with ice-cold PBS and lysed in 50 mM Tris (pH 7.5), 150 mM sodium chloride, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 0.07 trypsin inhibitor units/ml of aprotinin, and 1 μg/ml each of leupeptin, pepstatin, antipain, and chymostatin) (www.sigma-aldrich.com) (40). Lysate protein concentration was determined using the Bradford method (www.bio-rad.com).

Immunoprecipitation and immunoblotting:

These procedures have been described previously (14).

Confocal immunofluorescence microscopy:

Cells seeded on glass coverslips were stimulated with EGF as described above. Cell fixation and immunostaining was described previously (17). Cells were stained with the appropriate primary antibody (4 μg/ml anti-EGFR mAb 528 or 1:500 anti-LAMP-1 pAb in blocking buffer) and followed with 1:300 dilution of goat anti-mouse IgG (H+L) F(ab')2-Cy3 and anti-rabbit IgG F(ab')2-Cy2. Coverslips were mounted on glass slides using Fluoromount-G (www.emsdiasum.com). Confocal microscopy was carried out using a Leica TCS-NT Confocal Laser Scanning microscope fitted with krypton and argon lasers, as previously described (41).

Assessment of EGFR internalization:

Cells were grown on 10-cm tissue culture dishes to 70-80% confluence. Following serum starvation for 4-6h, the cells were incubated with 25 ng/ml Alexa-Fluor-488-conjugated EGF at 4°C for 30min, washed 3 times with cold PBS, and incubated at normal cell growth temperature for the indicated time points to allow internalization. The cells were placed on ice to stop internalization, rinsed 3 times with cold PBS and subjected to an acid wash (0.2 M acetic acid and 0.5 M NaCl, pH 2.8) for 5min. Non-internalized EGF was removed by 3 washes with PBS, and the cells were detached from tissue culture dishes using a rubber scraper. Cells were washed and suspended in FACS buffer (2% FBS and 0.01% sodium azide in PBS), and fixed by adding an equal volume of 4% formaldehyde/PBS. Fluorescence emission due to internalized EGF was detected by flow cytometry. Mean fluorescence intensity of cells after EGF binding but without the acid wash was set to 100%, percentage internalization was calculated after subtracting background (fluorescence of cells subjected to acid wash without allowing

internalization). Flow cytometry, data collection and analysis were performed on a FACSort machine using CellQuest software (Becton Dickinson, Franklin Lakes, NJ). Each experiment was done in triplicate 3 times. Values are expressed as a percentage of initial EGF binding. The data was analyzed statistically with Prism (Graphpad Software, Inc.) by using student t-test and one-way ANOVA. All of the values from a representative experiment are plotted. The median is connected by a line, and the range from maximum to minimum is expressed as an error bar.

Downregulation of cell-surface EGFR:

Downregulation of EGFR from the cell surface was assessed as previously described (17).

Results and Discussion

Ligand-induced EGFR ubiquitinylation and degradation are impaired in cells lacking endogenous Cbl expression: Previous studies supporting a role for Cbl in EGFR ubiquitinylation and downregulation have exclusively used overexpression of wildtype Cbl or its dominant negative mutants (17,18,42). However, the role of endogenous Cbl in EGFR ubiquitinylation and degradation has not been addressed directly. To address this question, we derived two distinct pairs of $Cbl^{+/+}$ and $Cbl^{-/-}$ mouse embryo fibroblast cell lines (MEFs), using $Cbl^{-/-}$ mouse lines developed independently in the Bowtell and Gu laboratories (34,35); these cell lines are designated $Cbl^{+/+}$ (DB) and $Cbl^{-/-}$ (DB), and $Cbl^{+/+}$ (HG) and $Cbl^{-/-}$ (HG), respectively.

Western blotting of whole cell lysates confirmed the Cbl protein expression in the $Cbl^{+/+}$ MEFs but not in either of the $Cbl^{-/-}$ MEFs (Fig. 1A). The $Cbl^{-/-}$(DB) MEFs express low levels of a truncated Cbl protein (data not shown) representing a nonfunctional splice product, as previously reported (34,37). Since other Cbl family members, such as Cbl-b and Cbl-c, may also play a role in EGFR downregulation (43,44), we wished to determine the level of expression of Cbl-b and Cbl-c in these MEFs. To assess the expression of Cbl-b, we used an antibody (H454) that recognizes both Cbl and Cbl-b (determined using HA-tagged Cbl and Cbl-b proteins; data not shown). We found that Cbl-b protein was detectable in the lysates of $Cbl^{+/+}$ and $Cbl^{-/-}$ MEFs from which all Cbl protein had been immunodepleted using a Cbl-specific antibody (Fig. 1B, lanes 5-8); notably, the level of Cbl-b protein was substantially lower in the $Cbl^{-/-}$ (HG) MEFs. We also assayed for the presence of Cbl-c by Northern blot, as no antibody is currently

available; the Cbl-c mRNA levels were nearly undetectable in all MEFs and there was no compensatory increase in $Cbl^{-/-}$ cells (data not shown). While the reasons for reduced Cbl-b expression in the $Cbl^{-/-}$ (HG) MEFs are unknown, this trait was advantageous to assess the contribution of endogenous Cbl to EGFR downregulation. To analyze the impact of Cbl deficiency on EGFR downregulation, we used retroviral infection to derive stable transfectants of the $Cbl^{+/+}$ and $Cbl^{-/-}$ MEFs expressing human EGFR on the cell surface (Fig. 1C and 1D).

As Cbl is an EGFR-directed ubiquitin ligase (10-12), we first assessed the ligand-induced EGFR ubiquitinylation in EGFR-expressing $Cbl^{+/+}$ and $Cbl^{-/-}$ MEFs. Serum-starved cells were stimulated with EGF for various times, and anti-EGFR immunoprecipitates (IPs) were subjected to immunoblotting (IB) with an anti-ubiquitin antibody. As anticipated, ligand-dependent ubiquitinylation of EGFR was readily detected in both $Cbl^{+/+}$ MEF lines (DB and HG; Fig. 2A). Notably, ligand-induced EGFR ubiquitinylation in both $Cbl^{-/-}$ MEF lines was greatly reduced, indicating that endogenous Cbl is the major ubiquitin ligase involved in EGFR ubiquitinylation in MEFs. Importantly, anti-EGFR blotting of anti-EGFR IPs (Fig. 2A, second panel) or whole cell lysates (Fig. 2B) revealed that the ligand-induced loss of EGFR protein was substantially slower in both $Cbl^{-/-}$ MEFs; concomitantly, we observed a slower and less pronounced loss of phosphotyrosine (pY) signals on EGFR (Fig. 2A, bottom panel). The pattern of EGFR phosphorylation also indicates that impaired EGFR ubiquitinylation in $Cbl^{-/-}$ cells is not due to defective EGFR activation.

To establish that defective EGFR ubiquitinylation and degradation in $Cbl^{-/-}$ MEFs was due to the lack of Cbl expression, and not an artifact of cell line derivation, we

reconstituted Cbl expression in the EGFR-expressing $Cbl^{-/-}$ (HG) MEFs. IB of whole cell lysates demonstrated that retrovirus-mediated introduction of HA-tagged Cbl led to reconstitution of Cbl expression in $Cbl^{-/-}$ (HG) MEFs (Fig. 2C). Comparison of the HA-Cbl-transfected versus the vector-transfected cells demonstrated that reconstitution with Cbl fully restored the EGF-induced ubiquitinylation and degradation of EGFR (Fig. 2D and 2E). Thus, the defect in EGFR ubiquitinylation and degradation in $Cbl^{-/-}$ (HG) MEFs is solely due to lack of Cbl expression. Overall, the results with $Cbl^{-/-}$ MEFs and their Cbl-reconstituted derivatives establish a clear role for endogenous Cbl in ligand-induced EGFR ubiquitinylation and degradation.

EGFR internalization is unaltered in $Cbl^{-/-}$ MEFs: The precise site(s) of Cbl action in the endocytic trafficking of EGFR has not been clarified. Based on the formation of a Cbl-CIN85-endophilin complex, a recent set of studies concluded that Cbl plays an important role in the initial internalization of EGFR (25,45). In addition, an EGFR mutant unable to bind Grb2 was impaired in its ability to form an EGFR-Grb2-Cbl complex, and to undergo ligand-induced ubiquitinylation, and internalization, further suggesting a role for Cbl-mediated ubiquitinylation in EGFR internalization (26). However, whether the effects of the CIN85 mutant are due to the loss of a Cbl-CIN85 interaction or of other protein-protein interactions, or whether Cbl-EGFR interaction through Grb2 is required for EGFR internalization, have not been established. In fact, the N-terminal half of Cbl, which included only the TKB and RING finger domains and lacked both the CIN85 and Grb2 interaction sites, was sufficient to enhance the ubiquitinylation and degradation of EGFR (17,46,47). Moreover, overexpression of Cbl

did not enhance the EGFR internalization (42), and dominant-negative Cbl mutants blocked ubiquitinylation and degradation but not the internalization of EGFR (17,18). These discrepancies suggest that the impairment of EGFR internalization by the dominant interfering forms of CIN85 may not be through an interruption of the Cbl-CIN85 interaction but through another mechanism. Similarly, Cbl-Grb2 interaction may not be the basis for Grb2-regulated EGFR internalization, as Grb2 can also recruit a number of other proteins, such as RN-tre, to activated EGFR (48). To clarify the role of Cbl in EGFR internalization, we used the MEF system characterized above to directly establish if the loss of Cbl function had an obvious impact on ligand-induced internalization of EGFR. For this purpose, we assayed the acid-stable uptake of Alexa-Fluor-488-conjugated-EGF by MEFs.

To minimize the contribution of recycling and/or lysosomal degradation of internalized EGFR, we quantified the Alexa-488-EGF uptake in MEFs for relatively brief time periods (up to 10 min). We also used EGF at a relatively low concentration (25 ng/ml) that only saturates 40-50% of cell surface EGFR in our system (data not shown). The rate of initial EGFR internalization in $Cbl^{-/-}$ (HG) MEFs was comparable to that in $Cbl^{+/+}$ (HG) MEFs (Fig. 3A, $P>0.05$). Furthermore, the rate of initial internalization of EGFR in Cbl-reconstituted and vector-transfected $Cbl^{-/-}$ (HG) MEFs was essentially identical (Fig. 3C, left panel, $P>0.05$), even though the downregulation of EGFR at later time points, which reflects a net balance of internalization, recycling and lysosomal degradation, was dramatically slower in $Cbl^{-/-}$ (HG) MEFs compared to Cbl-reconstituted MEFs (Fig. 3C, right panel, $P<0.001$). Unimpaired EGFR internalization in $Cbl^{-/-}$ (HG) MEFs was further confirmed by the immunofluorescence staining of EGFR after EGF

stimulation. The pattern of EGFR-staining endocytic vesicles 10 min after EGF stimulation was comparable in $Cbl^{-/-}$ and $Cbl^{+/+}$ (HG) MEFs (Fig. 3B).

The combination of unimpaired initial internalization and reduced EGFR degradation (and downregulation) in $Cbl^{-/-}$ MEFs indicates that Cbl function is critical at a post-internalization step in EGFR downregulation but not at the internalization step.

CHO-Ts20 cells demonstrate that the ubiquitin pathway is essential for EGFR degradation but dispensable for internalization: Previous analyses using overexpression of wildtype or mutant forms of Cbl have demonstrated a tight correlation between the Cbl-regulated ubiquitinylation and degradation of EGFR (49). This correlation is further strengthened by studies of EGFR mutants unable to bind to Cbl (50), and analyses of Cbl-deficient MEFs presented above. However, the questions of whether Cbl-mediated EGFR degradation proceeds through ubiquitinylation and whether the ubiquitin pathway is required to target EGFR for lysosomal degradation have not been directly addressed. To clarify these issues, we utilized a CHO cell line with a temperature-sensitive ubiquitin-activating (E1) enzyme, which results in a conditional defect in protein ubiquitinylation (51).

As CHO cells lack EGFR, the CHO cell line with mutant E1 (CHO-Ts20) and its wildtype counterpart (CHO-E36) were stably transfected with human EGFR. Transient overexpression of wildtype Cbl, in comparison with vector control, enhanced the ubiquitinylation of EGFR in Ts20-EGFR cells (Fig. 4A, compare lanes 2 and 4). In contrast, the overexpression of a Cbl RING finger domain mutant, Cbl-C3AHN, suppressed the ligand-induced ubiquitinylation (Fig. 4A, lane 6) and degradation of

EGFR (Fig. 4B, lanes 7-9). Thus, both ubiquitinylation and degradation of EGFR in CHO-Ts20 cells are dependent on endogenous Cbl.

To directly assess the impact of inhibiting ubiquitinylation in CHO-Ts20 cells on EGFR degradation, we compared Ts20-EGFR and E36-EGFR (control) cell lines at 30°C, the permissive temperature for ubiquitinylation, versus 42°C, the non-permissive temperature. When assayed at 30°C, the ligand-induced EGFR ubiquitinylation was observed in both Ts20-EGFR and E36-EGFR cells (Fig. 5A and 5B, lanes 1-3 and 7-9). In contrast, ligand-induced EGFR ubiquitinylation was markedly attenuated in Ts20-EGFR cells shifted to 42°C (Fig. 5A, lanes 10-12); as a control, the ubiquitinylation of EGFR in E36-EGFR cells was unaffected by the temperature shift (Fig. 5A, lanes 4-6). Analysis of EGFR protein levels over a longer time course of EGF stimulation demonstrated that the kinetics of EGFR degradation in E36-EGFR cells was similar at 30°C versus 42°C (Fig. 5B, compare lanes 1-4 and 5-8). In contrast, the degradation of EGFR in Ts20-EGFR cells was retarded when these cells were shifted to 42°C to block ubiquitinylation (Fig. 5B, compare lanes 9-12 and 13-16). The requirement of Cbl for EGFR degradation, together with the inhibition of EGFR degradation upon blockade of the ubiquitinylation pathway in Ts20-EGFR cells, supports the view that it is the ubiquitin ligase activity of Cbl rather than another function that is essential in Cbl-mediated EGFR degradation. Interestingly, in both $Cbl^{-/-}$ MEF cell lines that we utilized, we observed a low level of residual EGFR ubiquitinylation upon ligand stimulation. Whether this is due to a low level of Cbl-b expression or due to an unrelated ubiquitin ligase capable of targeting EGFR for ligand-induced ubiquitinylation, remains to be investigated. The residual ubiquitinylation could account for the EGFR degradation that

is still observed. Alternatively, the continued EGFR degradation could reflect the contribution of other mechanisms, such as those mediated by the dileucine motifs, to endosomal sorting of EGFR (52).

Our finding that the initial internalization of EGFR was intact in $Cbl^{-/-}$ MEFs suggested that Cbl and Cbl-mediated EGFR ubiquitinylation was dispensable for EGFR internalization, but left open the possibility that ubiquitinylation of another protein(s) may mediate the internalization. In this regard, EGF-inducible ubiquitinylation of endocytic proteins, such as Eps15 (53) and CIN85 (54), has been previously demonstrated, although the role of the ubiquitinylation in EGFR internalization remains to be established. Ubiquitin has been clearly recognized as a receptor internalization motif in yeast (20,21), while studies of the growth hormone receptor in mammalian cells indicate that ubiquitinylation of an unknown non-receptor component may be crucial for internalization (22). The Ts20-EGFR system provided a suitable system to test if the ubiquitin pathway was also essential for initial internalization of EGFR. Therefore, we also examined the EGFR internalization in Ts20-EGFR and E36-EGFR cells, using the assay described above. Similar to control E36-EGFR cells (Fig. 6, left panel, $P>0.05$), the rate of initial EGFR internalization in Ts20-EGFR cells grown at 30°C and 42°C was comparable (Fig. 6B, right panel, $P>0.05$). Thus, the ubiquitinylation machinery is dispensable for the initial internalization step in the endocytic traffic of EGFR.

Our results indicate that neither EGFR ubiquitinylation nor the monoubiquitinylation of other proteins, such as Eps15 and CIN85, is required for EGFR internalization. Dikic and colleagues have, however, observed that a dominant negative mutant of CIN85, which is not monoubiquitinylated by Cbl upon EGF stimulation,

impairs the internalization and degradation of EGFR (23, 45). It is likely that the dominant negative form of CIN85 fails to interact with other partners, which may relate to its role at the internalization step. Furthermore, Dikic and colleagues observed that the Cbl-dependent monoubiquitinylation of CIN85 primarily occurred after EGFR endocytosis (23), which suggests a role for ubiquitinylated CIN85 at a post-endocytic step, consistent with our conclusions.

Our results do not exclude the possibility that ubiquitinylation can function as an endocytic signal. In fact, monoubiquitinylation of EGFR is an enough signal for EGFR internalization (55). Our results do indicate, though, that this cannot be the sole mechanism for initial internalization, and that other mechanisms can fully support the internalization process in the absence of the ubiquitinylation of EGFR and any accessory proteins. The molecular nature of these additional signals remains to be fully elucidated. Several endocytic motifs have been identified in EGFR, including the dileucine-based motif (56) and the tyrosine-based AP-2 binding motif (57). However, mutational analyses suggest that none of these motifs is essential for internalization (56,57). It is likely that a complex RTK such as EGFR has evolved multiple redundant mechanisms to ensure internalization, as this is a key regulatory process.

The internally controlled Ts20-EGFR and E36-EGFR cell pair also provided an opportunity to assess the nature of the endocytic compartment(s) where Cbl functions and where ubiquitinylation plays a decisive role in the endocytic trafficking of internalized EGFR. For this purpose, we assessed the colocalization of the internalized EGFR with selected endocytic markers, using confocal microscopy. In both E36-EGFR and Ts20-EGFR cells grown at 30^{o}C, the EGFR-containing vesicles were peripherally distributed

at 5 min after EGF stimulation (Fig. 7A, B1 and E1) and gradually moved near the center of the cells, forming larger clusters by 30 min (Fig. 7A, C1 and F1). When E36-EGFR and Ts20-EGFR cells were compared at 42°C, the EGFR staining pattern at 5 min of EGF stimulation was similar (Fig. 7B, compare B1 and E1), consistent with the unaltered EGFR internalization seen in the internalization assay. Notably, however, the distribution of EGFR containing vesicles in Ts20-EGFR versus E36-EGFR cells grown at 42°C and stimulated for 30 min was quite distinct; these vesicles failed to move near the center and remained near the periphery in Ts20-EGFR cells (Fig. 7B, compare C1 and F1).

To characterize the EGFR-containing endosomal compartments, we either loaded the cells with fluorescent transferrin to mark the early/recycling endosomes, or carried out double staining for EGFR (Red) and LAMP-1 (36), a late endosome/lysosome marker; the cells were then analyzed using confocal microscopy. At 5 min of EGF stimulation at 30°C, the internalized EGFR in peripherally distributed vesicles mostly colocalized with transferrin (Fig. 7A, B3 and B4; E3 and E4; yellow); this colocalization was lost by 30 min of stimulation, when the EGFR was predominantly centrally clustered (Fig. 7A, C3 and C4; F3 and F4; red). However, EGFR in these vesicles colocalized with LAMP-1 (Fig. 8, B3 and B4; E3 and E4; yellow), indicating that a proportion of internalized EGFR underwent a time-dependent endocytic transport from early to late endosomes. This pattern remained unaltered when the E36-EGFR control cells were examined at 42°C (Fig. 7B, C3 and C4). In contrast, when Ts20-EGFR cells were grown at 42°C to disrupt ubiquitinylation, the internalized EGFR remained colocalized with transferrin at 30 min of EGF stimulation (Fig. 7B, F3 and F4; yellow), and showed a

lower degree of colocalization with LAMP-1 (Fig. 8, F3 and F4). These results indicate that ubiquitinylation is crucial for an early endosome to late endosome sorting step in the endocytic trafficking of ligand-stimulated EGFR.

The simplest interpretation of these results is that Cbl-mediated ubiquitinylation of EGFR (and/or other accessory proteins) serves as an endosomal sorting signal for EGFR delivery from early to late endosome/lysosome, and that ubiquitin modification serves as an essential signal at this step to ensure the efficient delivery of EGFR into the degradative compartments of the endocytic machinery. This model is consistent with the previous finding that ubiquitinylation and proteasome activity is needed for EGFR transfer into internal vesicle of MVBs (28). It is also compatible with the genetically defined role of ubiquitinylation of yeast transmembrane receptors for sorting into the inner vesicles of the MVB (58,59). In fact, yeast studies have identified endosomal sorting of receptor traffic (ESCRTs) complexes, such as ESCRT-1 (60), that function as ubiquitin recognizing proteins within the endocytic system. Deletion of TSG-101, one of the ESCRT-1 complex components in mammals, retards EGFR degradation and causes accumulation of ubiquitinylated proteins on endosomes (61). Furthermore, the hepatocyte growth factor receptor substrate (Hrs), the mammalian counterpart of the yeast ESCRT-2 protein also has a ubiquitin interacting motif (UIM) (53,62,63). A truncated Hrs protein lacking the UIM impaired EGFR degradation in *Drosophila*, by affecting endosome membrane invagination and MVB formation (64). Thus, Cbl-mediated ubiquitinylation could represent the receptor modification required for recognition by mammalian ESCRT proteins to facilitate endosomal sorting of EGFR to lysosomes. Whether or not the ubiquitin modification provides the signal for delivering

EGFR into the inner vesicles of the MVB in mammalian cells, and the regulatory control of this process, is an important area of future investigation.

In conclusion, our studies utilizing two distinct and independent experimental systems provide strong evidence that Cbl-mediated ubiquitinylation is essential for efficient ligand-induced degradation of EGFR, but is dispensable for initial receptor internalization. Future studies should address whether this dichotomy is a general feature of Cbl regulation of RTKs and other transmembrane receptors.

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Abbreviations:

CHO, Chinese hamster ovary; EGFR, epidermal growth factor receptor; IP, immunoprecipitate; IB, immunoblot; mAb, monoclonal antibody; LAMP, lysosome-associated membrane protein; MEF, mouse embryonic fibroblast; PAb, rabbit polyclonal antibody; PBS, phosphate-buffered saline; RTK, receptor tyrosine kinases; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; WT, wildtype.

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Figure legends:

Fig. 1. Characterization of $Cbl^{+/+}$ and $Cbl^{-/-}$ MEFs. A. Lack of Cbl expression in Cbl $^{-/-}$ MEFs. 50 μg aliquots of cell lysates of HG and DB MEFs were resolved by SDS-PAGE and subjected to IB with an anti-Cbl antibody. The faster migrating band in $Cbl^{-/-}$ (DB) cell line is a truncated nonfunctional protein. **B.** Expression of Cbl-b relative to Cbl in MEFs, analyzed with a Cbl/Cbl-b cross-reactive antibody H454. 1 mg aliquots of cell lysates were subjected to IP with an anti-Cbl-specific antibody (Santa Cruz C-15, lanes 1-4). Alternatively, the same amounts of lysates were first subjected to two rounds of anti-Cbl IP, and immunodepletion of Cbl was confirmed by IB (not shown). These immunodepleted lysates were subjected to IP with H454 antibody (lanes 5-8) to immunoprecipitate Cbl-b. Both sets of IPs were subjected to IB with H454 antibody (recognizes both Cbl and Cbl-b; data not shown). Note reduced Cbl-b signal in $Cbl^{-/-}$ (HG) MEFs (lane 8). **C and D.** Analysis of EGFR expression on $Cbl^{+/+}$ and $Cbl^{-/-}$ MEFs. $Cbl^{+/+}$ and $Cbl^{-/-}$ MEFs were stably transfected with EGFR, or vector (-) using retroviral infection. 50 μg aliquots of cell lysate proteins were immunoblotted with an anti-EGFR Ab (sc-03) (C). Alternatively, cells were trypsinized and stained with an anti-EGFR mAb (528, thin line) or an isotype control antibody (anti-Syk; thick line) followed by FACS analysis to quantify the cell surface expression of EGFR (D). The numbers in each box represent mean fluorescence intensity (arbitrary values) of anti-EGFR staining.

Fig. 2. Impairment of EGFR ubiquitinylation and degradation in $Cbl^{-/-}$ MEFs, and reversal of these defects by reconstitution of Cbl expression. A. Impaired EGFR

ubiquitinylation in $Cbl^{-/-}$ MEFs. The MEFs were stimulated with EGF (100 ng/ml) for the indicated times (min) prior to lysis. Anti-EGFR (mAb 528) IPs from 2mg aliquots of cell lysates were serially immunoblotted with anti-ubiquitin (upper panel), anti-EGFR (sc-03; middle panel) and anti-pY (4G10) (lower panel) antibodies. Ubiquitinylated EGFR is indicated. **B.** Delayed EGFR degradation in $Cbl^{-/-}$ MEFs. The cell lysates were prepared as in A, and 50 μg aliquots of cell lysates were immunoblotted with anti-EGFR antibody sc-03. The relative EGFR signals were quantified by densitometry using the Scion Image software (Scion Corp., Maryland), and are depicted as a proportion of signals observed with unstimulated cell lysates (set as 1). **C-E.** Reversal of defective EGFR ubiquitinylation and degradation in $Cbl^{-/-}$ MEFs by Cbl reconstitution. Retroviral infection was used to derive HA-Cbl and vector transfected $Cbl^{-/-}$ (HG) MEFs. 50 μg aliquots of lysates of these cells, as well as untransfected or vector-transfected $Cbl^{+/+}$ MEFs, were immunoblotted with an anti-Cbl antibody (C). The relative levels of Cbl expression, as determined by densitometry, are indicated at the bottom. The vector-transfected or Cbl-reconstituted $Cbl^{-/-}$ (HG) MEFs were stimulated with EGF for the indicated times prior to cell lysis. 2 mg aliquots of lysates were used for anti-EGFR IP followed by serial anti-ubiquitin (upper panel) and anti-EGFR immunoblotting (lower panel) (D). 50 μg aliquots of the same lysates were directly immunoblotted with anti-EGFR antibody (E) to assess EGFR degradation. Relative EGFR signals were determined by densitometry.

Fig. 3. Unimpaired internalization but reduced downregulation of EGFR in $Cbl^{-/-}$ MEFs. A. EGFR internalization in $Cbl^{+/+}$, $Cbl^{-/-}$ MEFs. The internalization assay is

described in Materials and Methods. **B.** Internalization of EGFR as determined by immunofluorescence staining as described in Materials and Methods. Cells were either left unstimulated or stimulated with EGF (25 ng/ml for 10min). Internalization is indicated by the accumulation of intracellular endocytic vesicles staining for EGFR (negative controls are not shown). **C.** EGFR internalization and downregulation in $Cbl^{-/-}$ and Cbl-reconstituted $Cbl^{-/-}$ MEFs. Internalization assay is described as in A. For downregulation, cells were either left unstimulated or stimulated with EGF as in B. The levels of EGFR on the cell surface were quantified by FACS analysis after immunostaining with anti-EGFR Ab 528 as described in Materials and Methods. EGFR levels remaining on the cell surface are represented as a percentage of EGFR levels (mean fluorescence intensity) without EGF stimulation. Every experiment was done in triplicate 3 times. All of the three values from one representative experiment are plotted. The median is connected by a line, and the range from maximum to minimum is expressed as an error bar.

Fig. 4. Ubiquitinylation and degradation of EGFR in CHO-Ts20 cells is mediated by Cbl. A. Wildtype Cbl enhances while the Cbl RING finger mutant inhibits EGFR ubiquitinylation. CHO-Ts20-EGFR cells were transiently transfected with the pAlterMAX vector or constructs encoding HA-Cbl or HA-Cbl-C3AHN (RING finger mutant). Cells were grown at 30^{o}C and either left unstimulated or stimulated for 10min prior to lysis. 1 mg aliquots of cell lysates were subjected to anti-EGFR IPs followed by serial anti-ubiquitin (top panel) and anti-EGFR (bottom panel) IB. **B.** Wildtype Cbl enhances while the Cbl-C3AHN inhibits EGFR degradation. Cell lysates at various times

following EGF stimulation were prepared as in A, and 50 μg aliquots of lysates were subjected to anti-EGFR IB. The relative EGFR signals (no EGF = 1) were determined by densitometry and are indicated at the bottom.

Fig. 5. Conditional impairment of EGFR ubiquitinylation and degradation in CHO-Ts20 cells. A. Lack of EGFR ubiquitinylation at 42°C in CHO-Ts20-EGFR cells. Control CHO-E36 cells (Left panel) and mutant E1-expressing CHO-Ts20 cells (Right panel) were stably transfected with human EGFR and clones expressing EGFR were identified by IB. The cells were plated at 30°C and then either continued at the same temperature (Lane 1-3 and 7-9) or shifted to 42°C (lane 4-6 and 10-12) for 4 hours, prior to stimulation with 100 ng/ml EGF for the indicated times and then lysed. 1 mg aliquots of cell lysates were subjected to anti-EGFR IPs and serial immunoblotting with anti-ubiquitin (top panel), anti-EGFR (middle panel) and anti-pY (bottom panel) Abs. **B.** Reduced EGFR degradation at 42°C in CHO-Ts20-EGFR cells. Cell lysates at various times following EGF stimulation were prepared as in A, and 50 μg aliquots of lysates were subjected to anti-EGFR IB. The relative EGFR signals (no EGF = 1) were determined by densitometry and are indicated at the bottom.

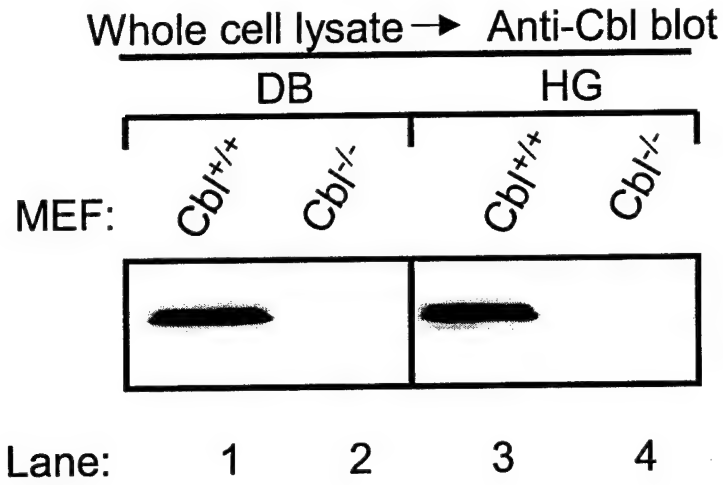
Fig. 6. Unimpaired EGFR internalization in CHO-Ts20 cells. Cells were grown at 30°C, and then either continued at the same temperature or shifted to 42°C, as indicated. Cells were allowed to bind Alexa-Fluor488-conjugated EGF at 4°C for 30min, and then returned to 37°C to allow EGF internalization. Every experiment was done in triplicate 3 times. The percentage of internalized EGF was calculated and plotted as in Fig. 3.

Fig. 7. Conditional alteration of internalized EGFR localization and its colocalization with transferrin receptor in CHO-Ts20 cells. Cells were either maintained at 30°C (A) or were shifted to 42°C (B), as in Fig. 6, and stimulated with EGF for the indicated times. Cells were loaded with Alexa-Fluor488-conjugated transferrin (36) for 5min prior to harvesting to visualize the early endosome/recycling endosome compartment. Cells were fixed, permeabilized and stained with anti-EGFR (mAb 528) followed by Cy3-conjugated anti-mouse antibody (red), and analyzed by confocal microscopy. Colocalization is indicated by yellow coloration in merged images.

Fig. 8. Conditional impairment of EGFR localization to late endosome/lysosome in CHO-Ts20 cells. Cells were processed as in Fig. 8 and stained with anti-EGFR antibody (visualized with Cy3-conjugated anti-mouse antibody; red) and rabbit anti-LAMP-1 antibody (late endosome/lysosome marker; visualized with Cy2-conjugated goat anti-rabbit antibody; green), and analyzed by confocal microscopy.

Fig. 1

A


Whole cell lysate → Anti-Cbl blot
DB
HG
MEF:
Cbl+/+
Cbl-/-
Cbl+/+
Cbl-/-
Lane:
1
2
3
4

B

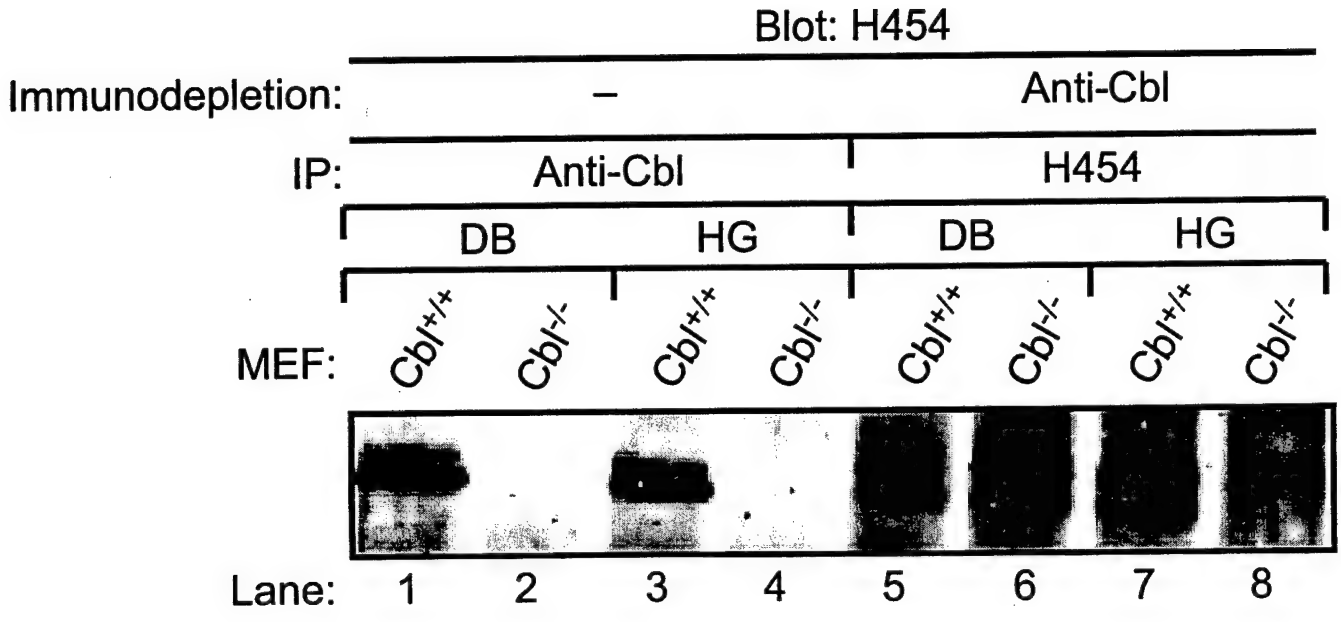
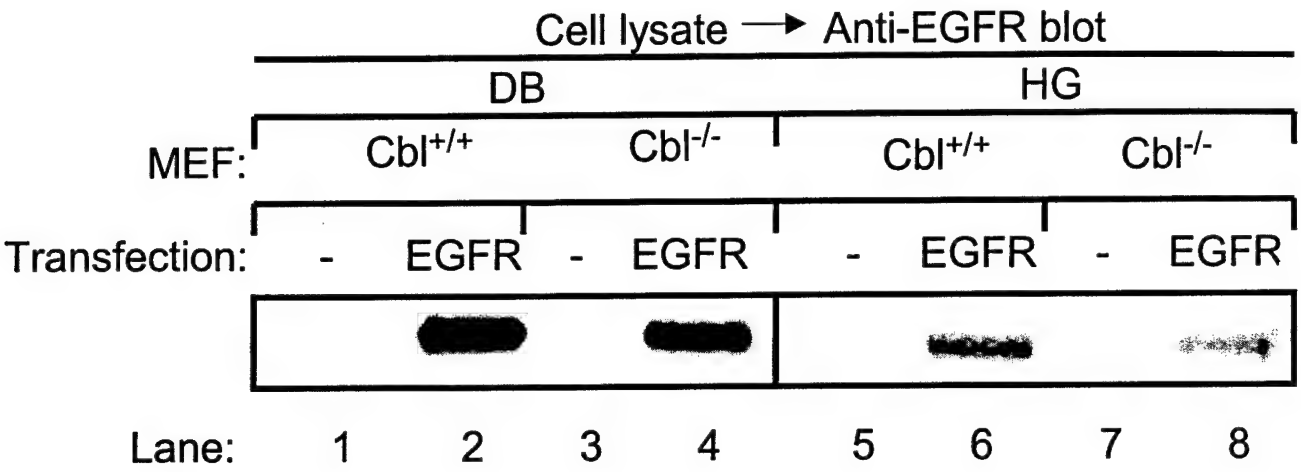

Blot: H454
Immunodepletion:
–
Anti-Cbl
IP:
Anti-Cbl
H454
DB
HG
DB
HG
MEF:
Cbl+/+
Cbl-/-
Cbl+/+
Cbl-/-
Cbl+/+
Cbl-/-
Cbl+/+
Cbl-/-
Lane:
1
2
3
4
5
6
7
8

Fig. 1-con't.

C


Cell lysate → Anti-EGFR blot
DB
HG
MEF:
Cbl+/+
Cbl-/-
Cbl+/+
Cbl-/-
Transfection:
-
EGFR
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EGFR
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EGFR
-
EGFR
Lane:
1
2
3
4
5
6
7
8

D

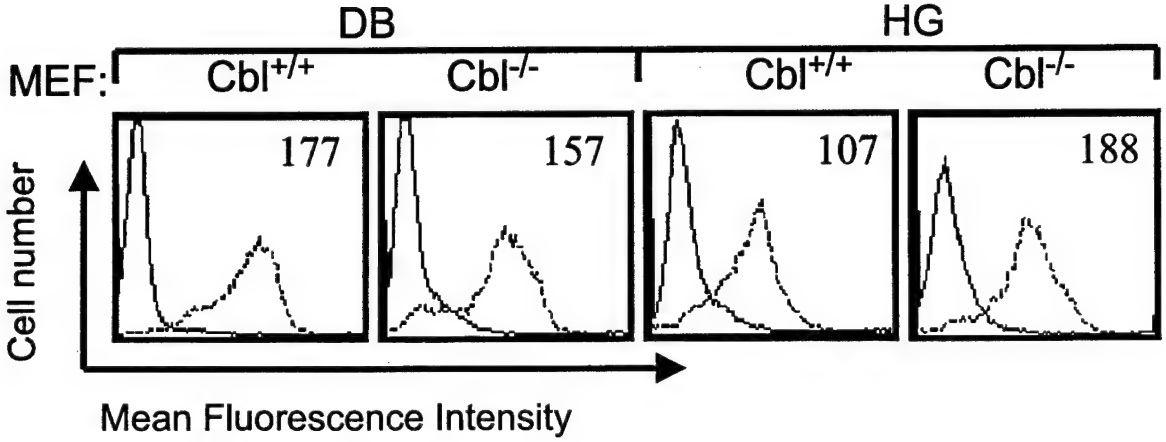
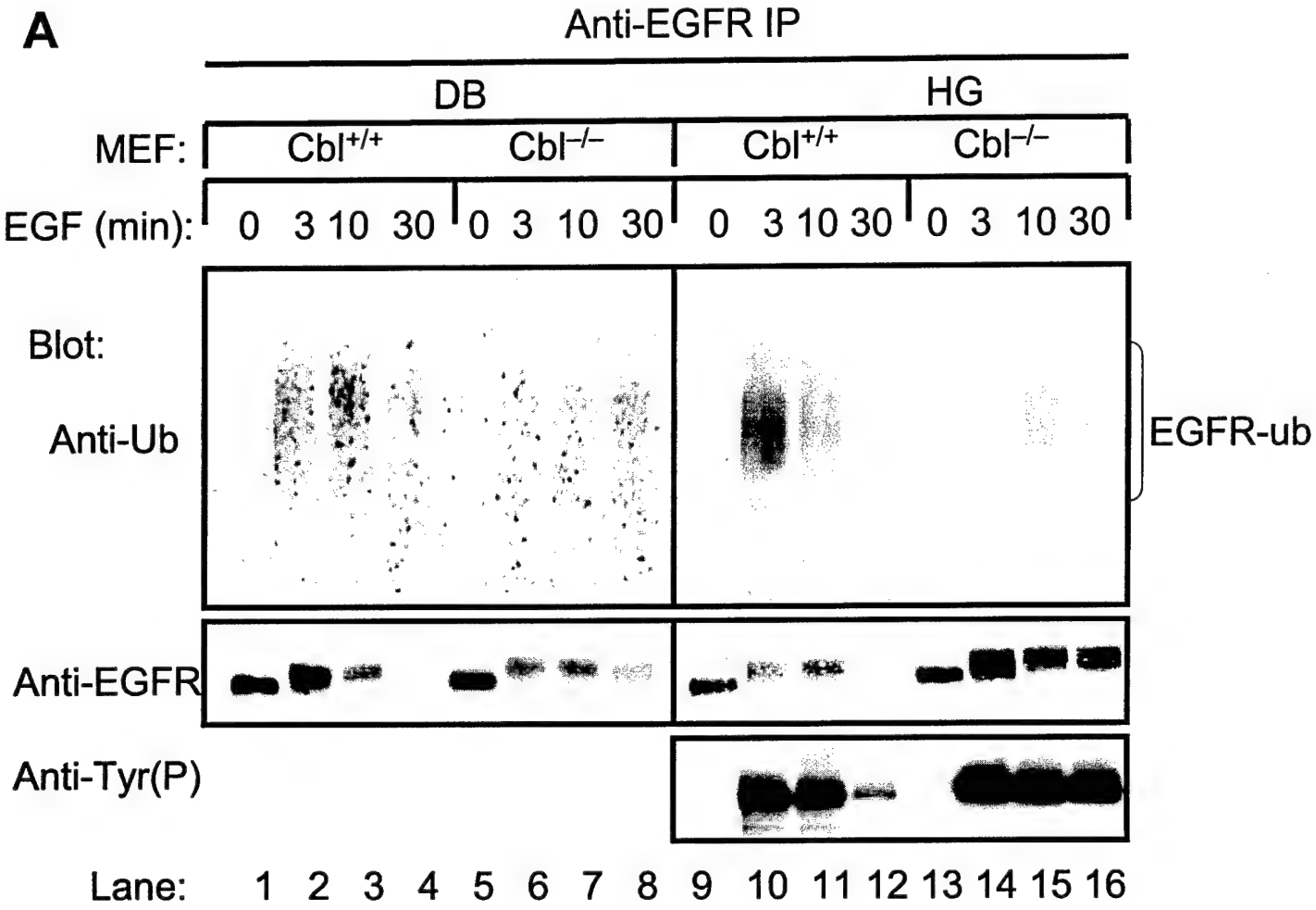

DB
HG
MEF:
Cbl+/+
Cbl-/-
Cbl+/+
Cbl-/-
177
157
107
188
Cell number
Mean Fluorescence Intensity

Fig. 2


A
Anti-EGFR IP
DB
HG
MEF:
Cbl+/+
Cbl−/−
Cbl+/+
Cbl−/−
EGF (min):
0 3 10 30 0 3 10 30 0 3 10 30 0 3 10 30
Blot:
Anti-Ub
EGFR-ub
Anti-EGFR
Anti-Tyr(P)
Lane:
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

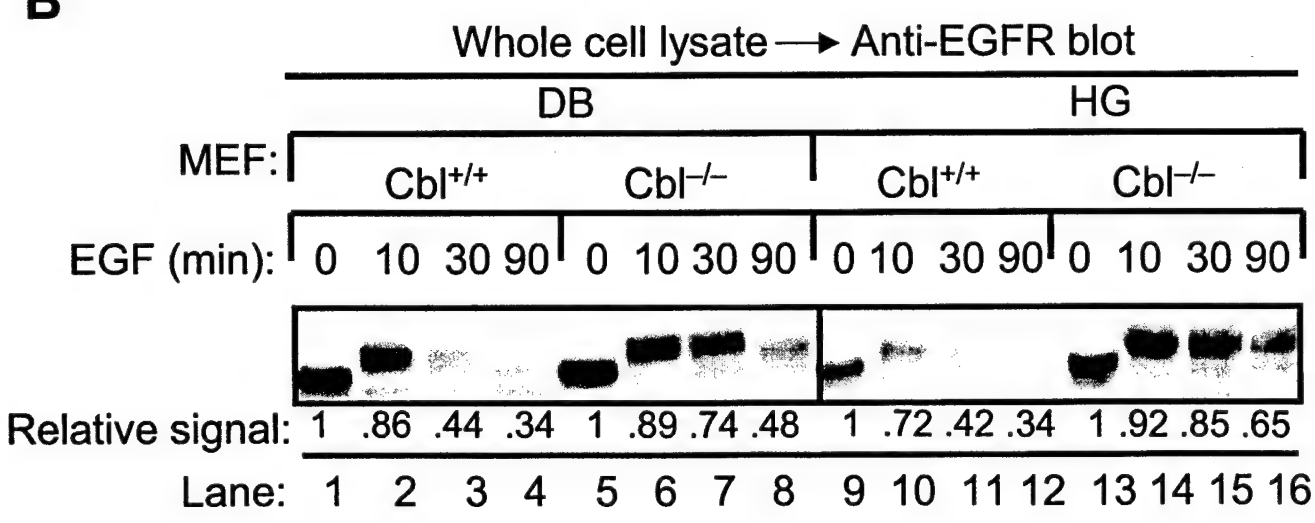
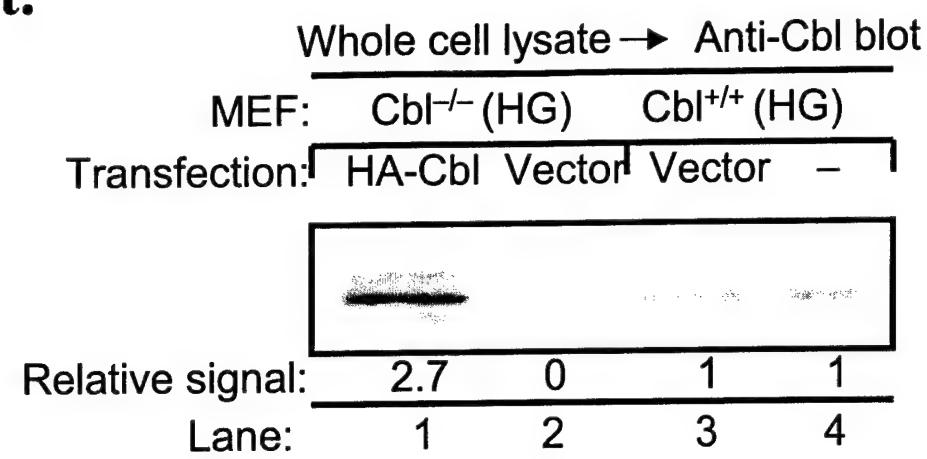

B
Whole cell lysate → Anti-EGFR blot
DB
HG
MEF:
Cbl+/+
Cbl−/−
Cbl+/+
Cbl−/−
EGF (min):
0 10 30 90 0 10 30 90 0 10 30 90 0 10 30 90
Relative signal:
1 .86 .44 .34 1 .89 .74 .48 1 .72 .42 .34 1 .92 .85 .65
Lane:
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Fig. 2-con't.

C



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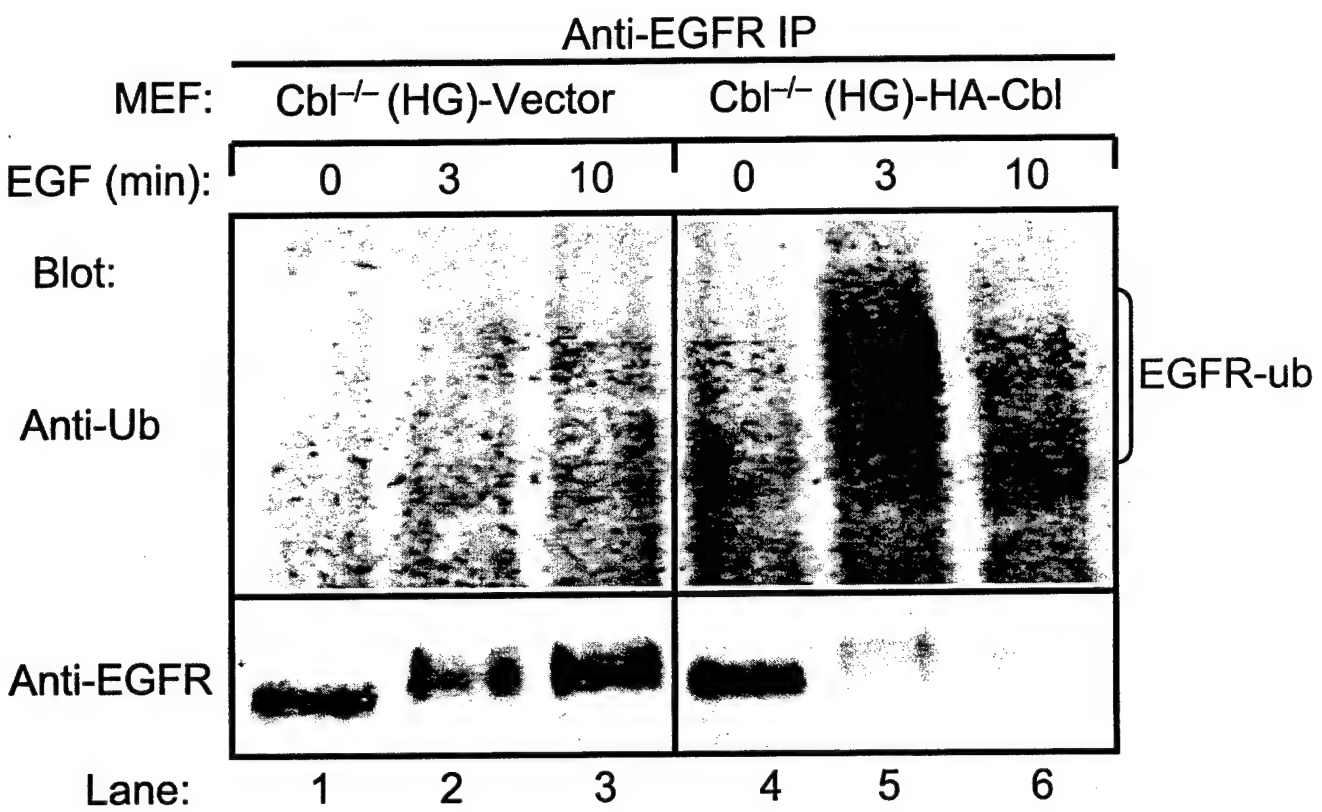


E

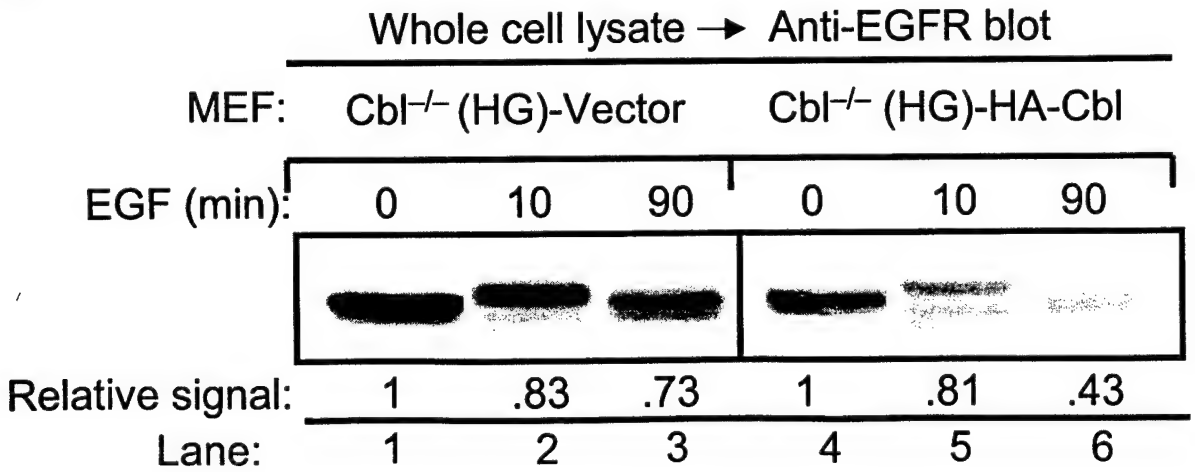
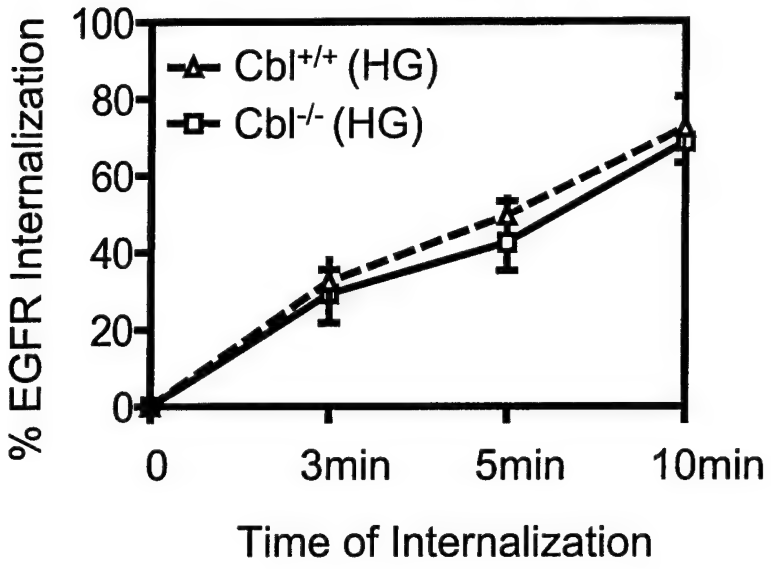


Fig. 3

A


% EGFR Internalization
100
80
60
40
20
0
Cbl+/+ (HG)
Cbl-/- (HG)
0
3min
5min
10min
Time of Internalization

B

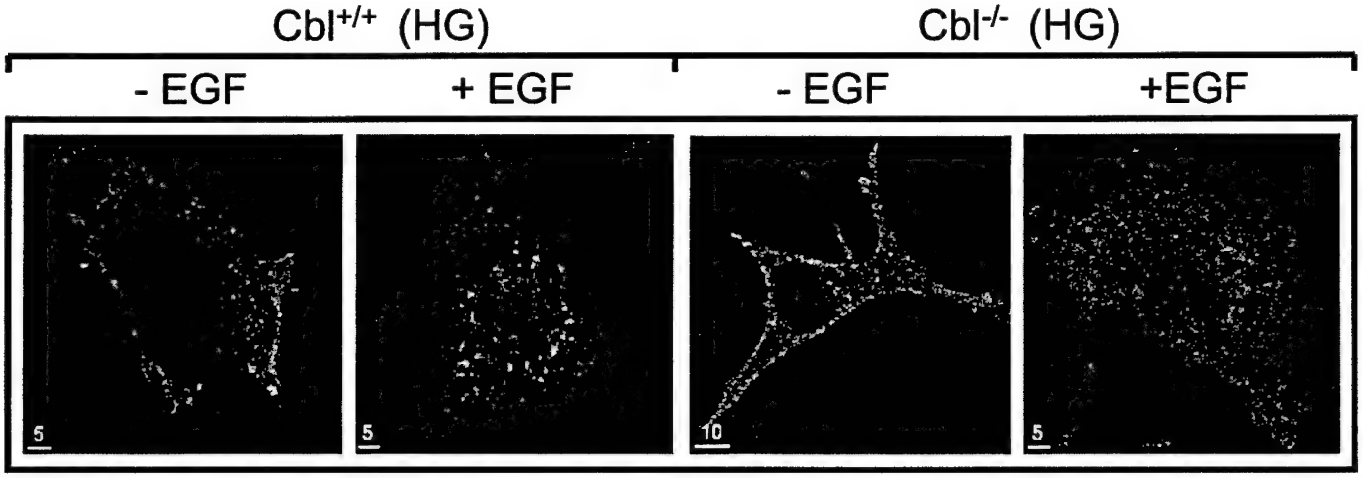

Cbl+/+ (HG)
Cbl-/- (HG)
- EGF
+ EGF
- EGF
+EGF
5
5
10
5

Fig. 3-con' t.

C

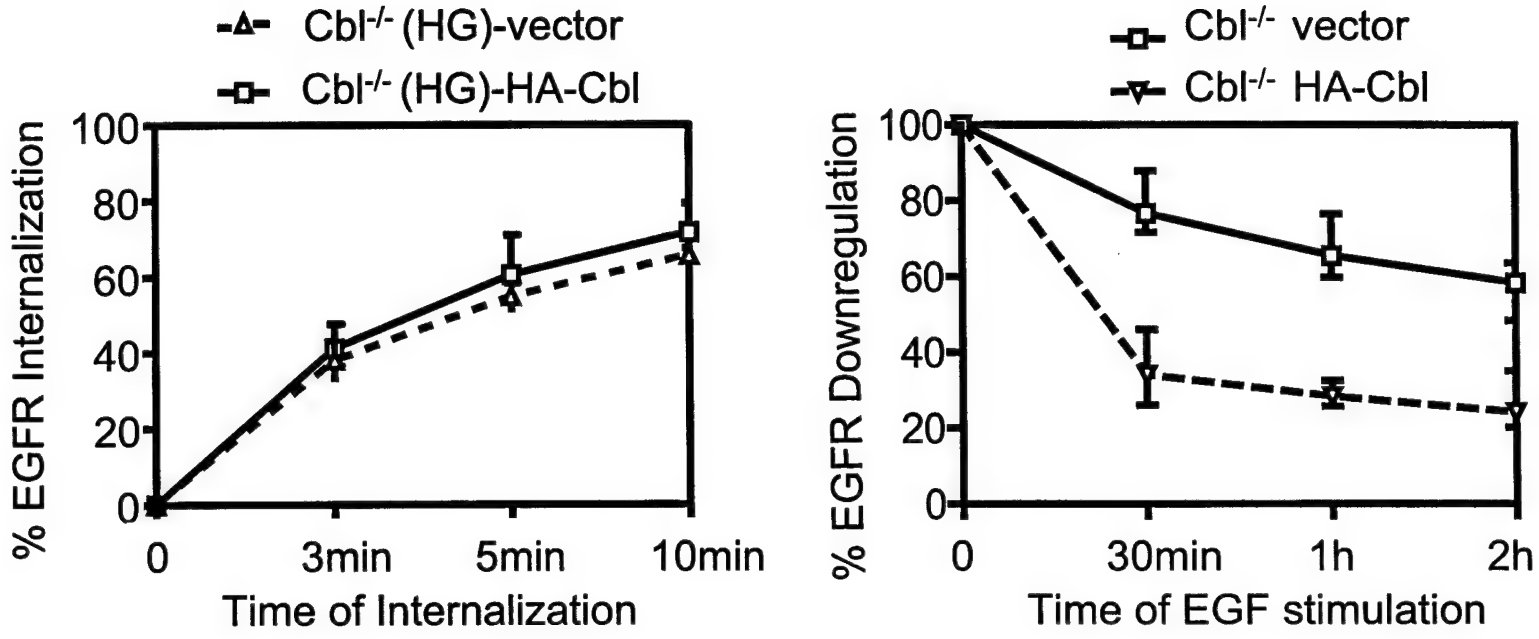
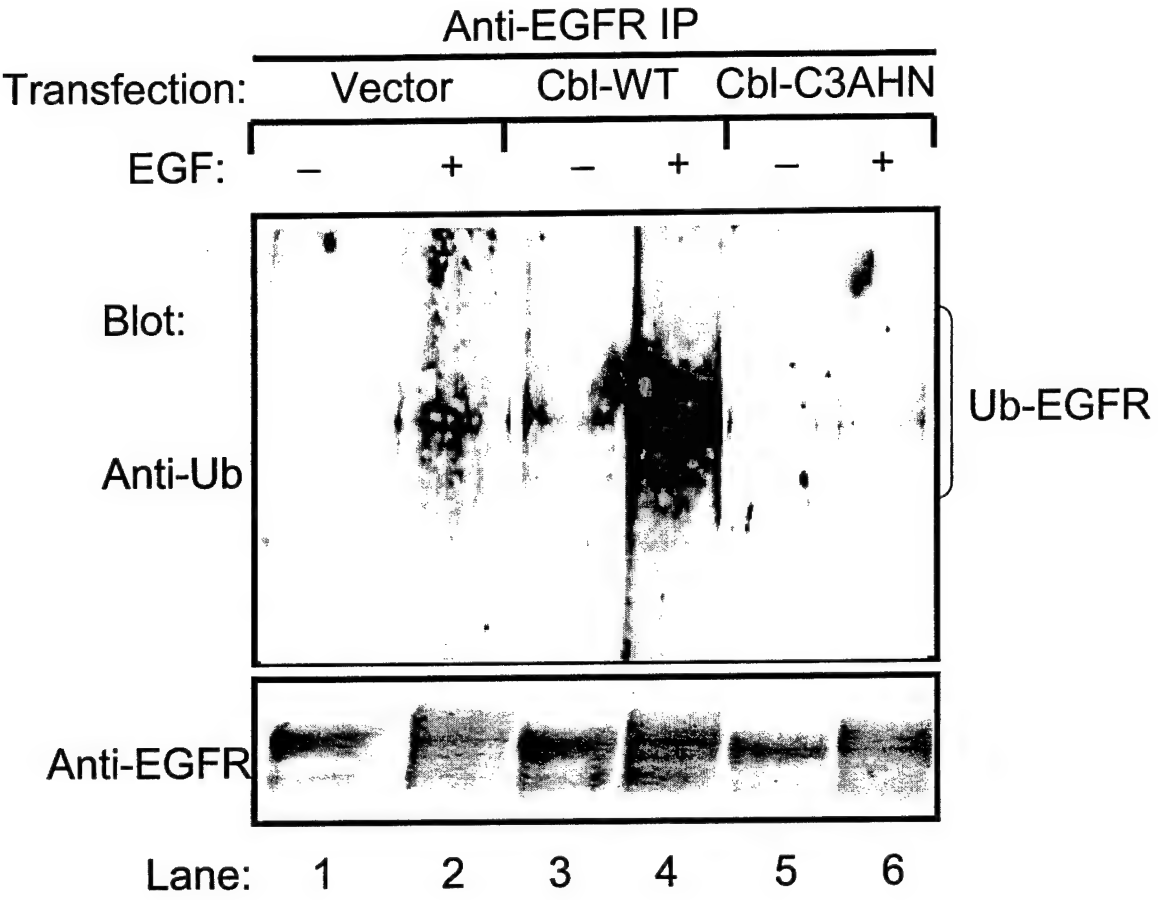

Cbl-/- (HG)-vector
Cbl-/- (HG)-HA-Cbl
% EGFR Internalization
100
80
60
40
20
0
0
3min
5min
10min
Time of Internalization
Cbl-/- vector
Cbl-/- HA-Cbl
% EGFR Downregulation
100
80
60
40
20
0
0
30min
1h
2h
Time of EGF stimulation

Fig. 4

A


Anti-EGFR IP
Transfection:
Vector
Cbl-WT
Cbl-C3AHN
EGF:
–
+
–
+
–
+
Blot:
Anti-Ub
Ub-EGFR
Anti-EGFR
Lane:
1
2
3
4
5
6

B

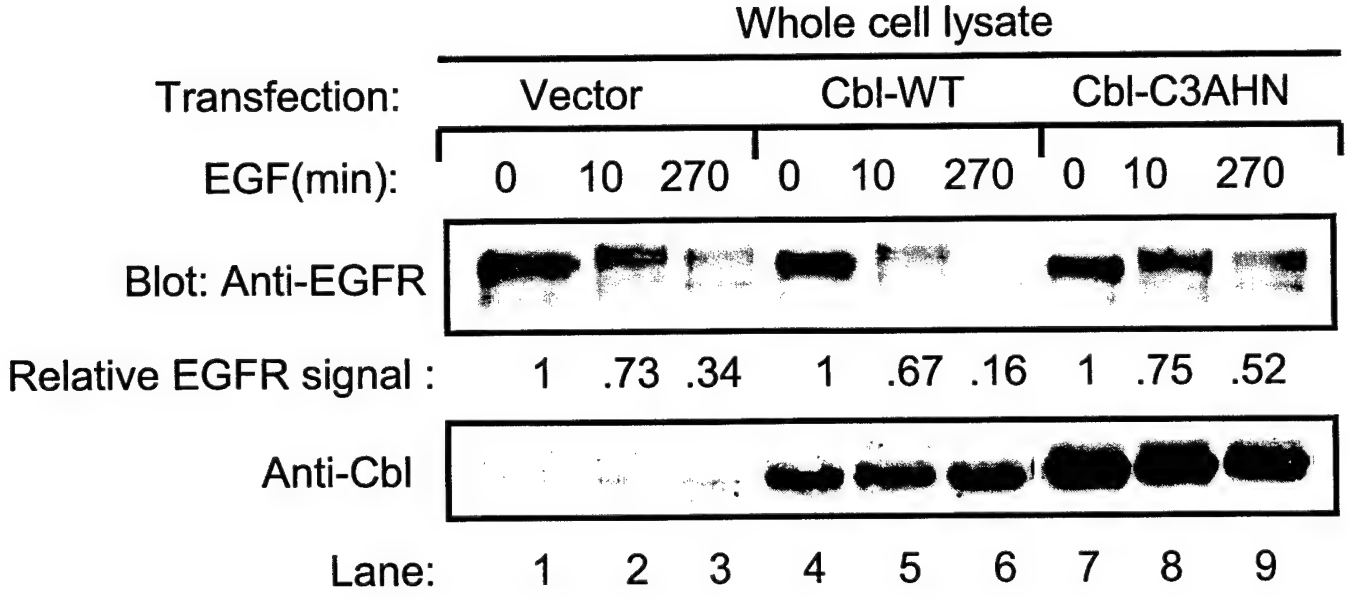

Whole cell lysate
Transfection:
Vector
Cbl-WT
Cbl-C3AHN
EGF(min):
0
10
270
0
10
270
0
10
270
Blot: Anti-EGFR
Relative EGFR signal :
1
.73
.34
1
.67
.16
1
.75
.52
Anti-Cbl
Lane:
1
2
3
4
5
6
7
8
9

Fig. 5 A

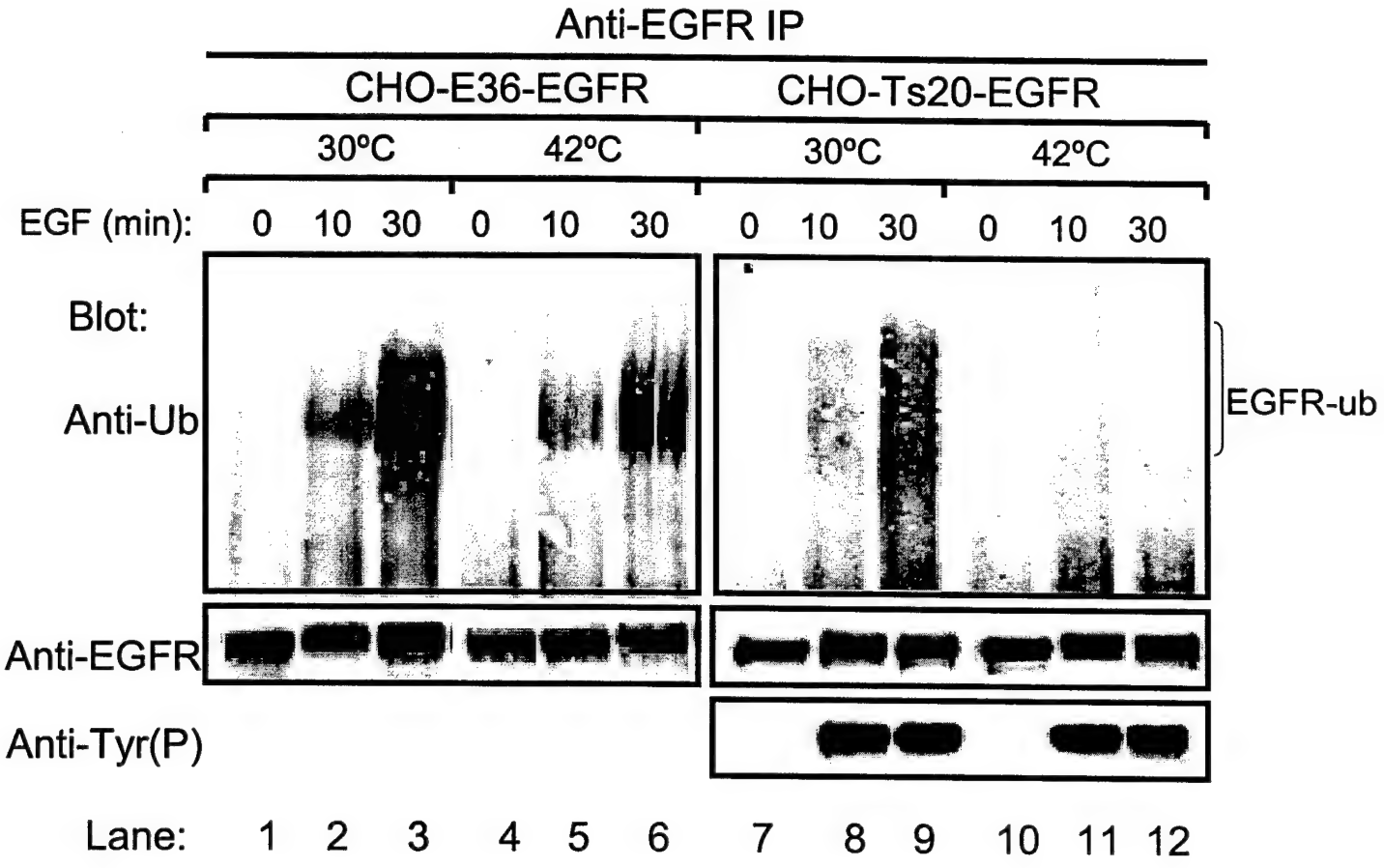

Anti-EGFR IP
CHO-E36-EGFR
CHO-Ts20-EGFR
30°C
42°C
30°C
42°C
EGF (min):
0 10 30 0 10 30 0 10 30 0 10 30
Blot:
Anti-Ub
EGFR-ub
Anti-EGFR
Anti-Tyr(P)
Lane:
1 2 3 4 5 6 7 8 9 10 11 12

Fig. 5 B

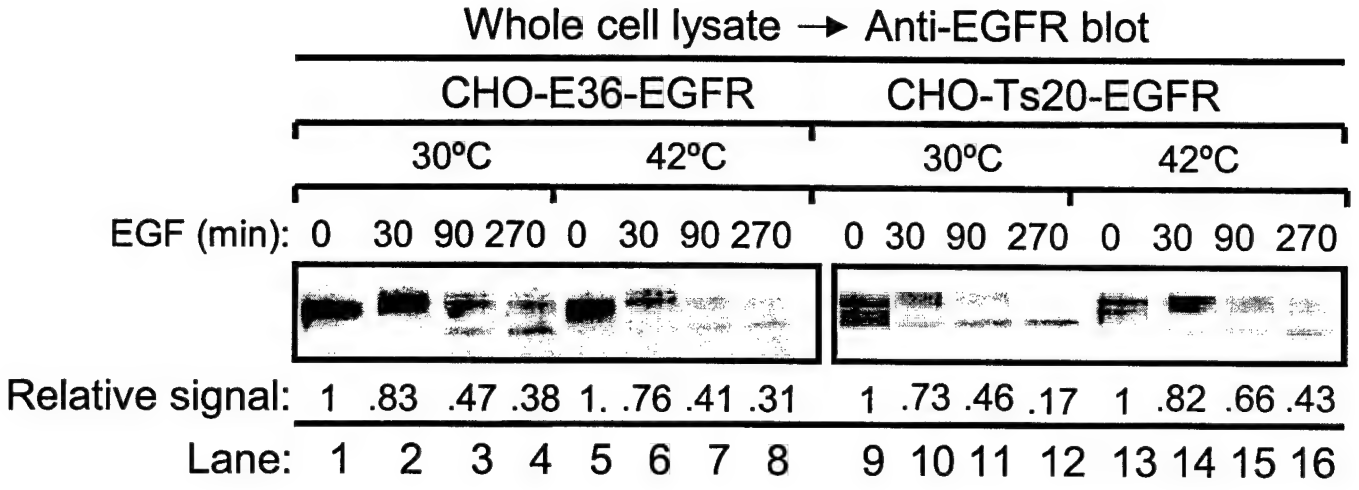

Whole cell lysate → Anti-EGFR blot
CHO-E36-EGFR
CHO-Ts20-EGFR
30°C
42°C
30°C
42°C
EGF (min): 0 30 90 270 0 30 90 270 0 30 90 270 0 30 90 270
Relative signal: 1 .83 .47 .38 1. .76 .41 .31 1 .73 .46 .17 1 .82 .66 .43
Lane: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Fig. 6

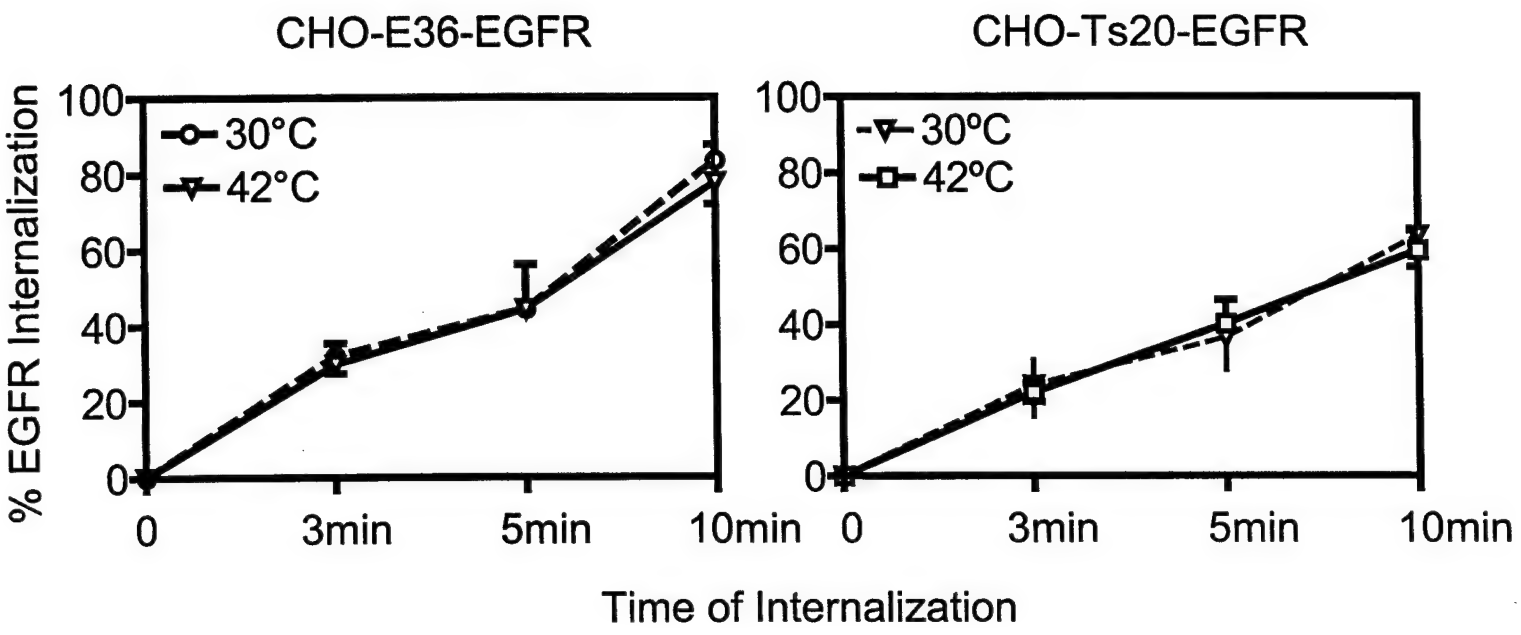

CHO-E36-EGFR
CHO-Ts20-EGFR
% EGFR Internalization
100
80
60
40
20
0
30°C
42°C
0
3min
5min
10min
Time of Internalization

Fig. 7A

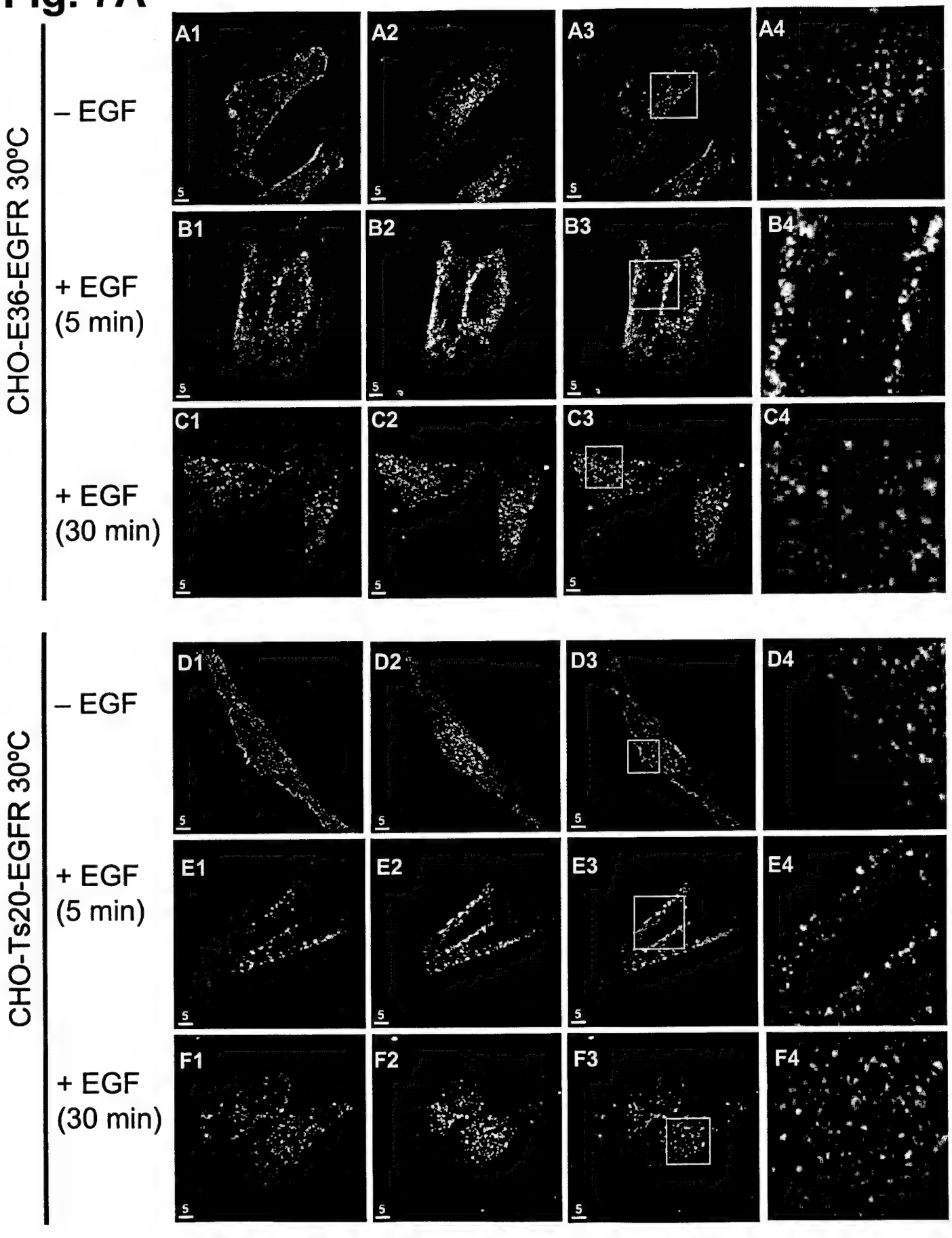

CHO-E36-EGFR 30°C
– EGF
+ EGF (5 min)
+ EGF (30 min)
A1
A2
A3
A4
B1
B2
B3
B4
C1
C2
C3
C4
CHO-Ts20-EGFR 30°C
– EGF
+ EGF (5 min)
+ EGF (30 min)
D1
D2
D3
D4
E1
E2
E3
E4
F1
F2
F3
F4
EGFR
Transferrin
Merge

Fig. 7B

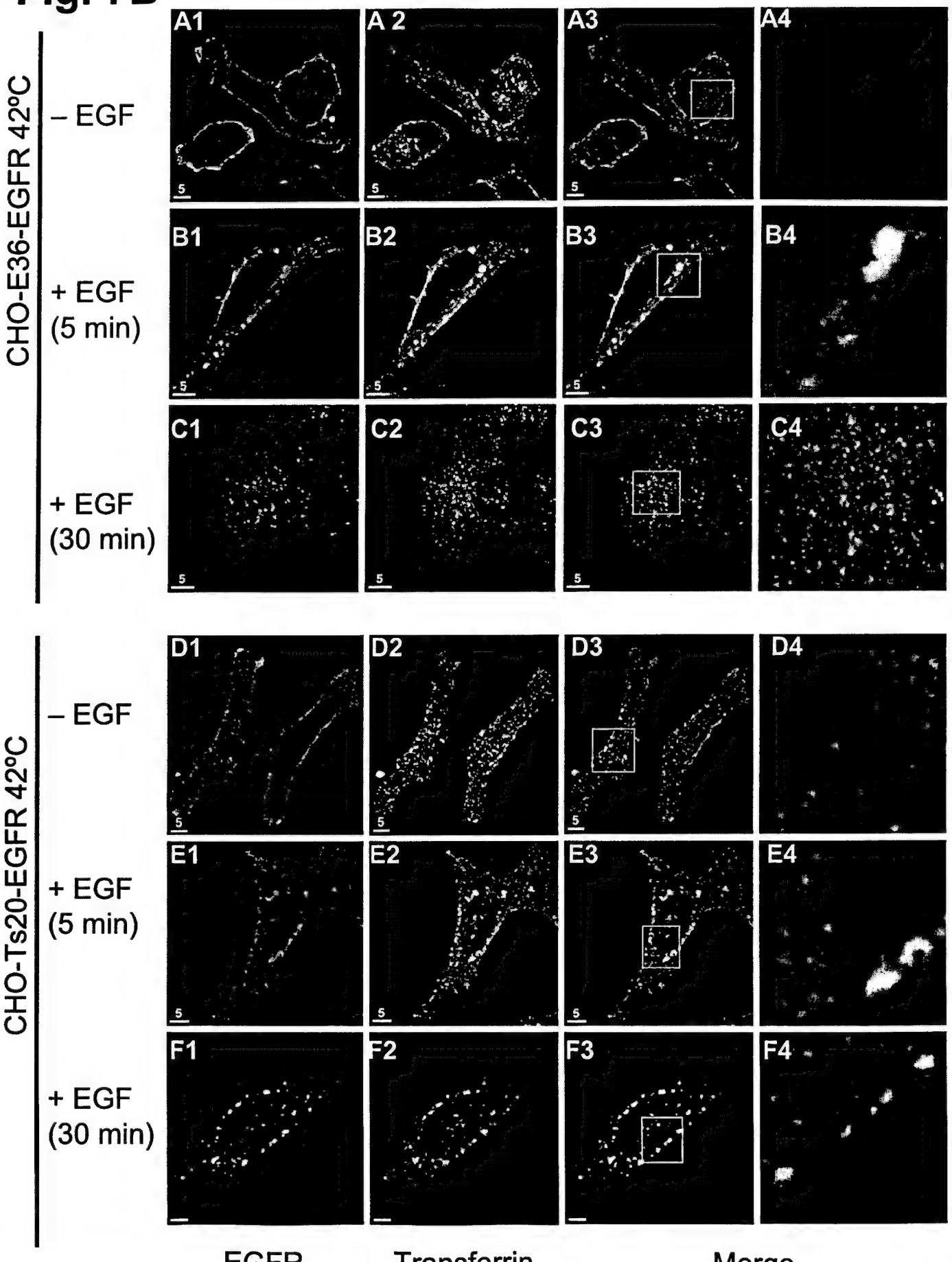
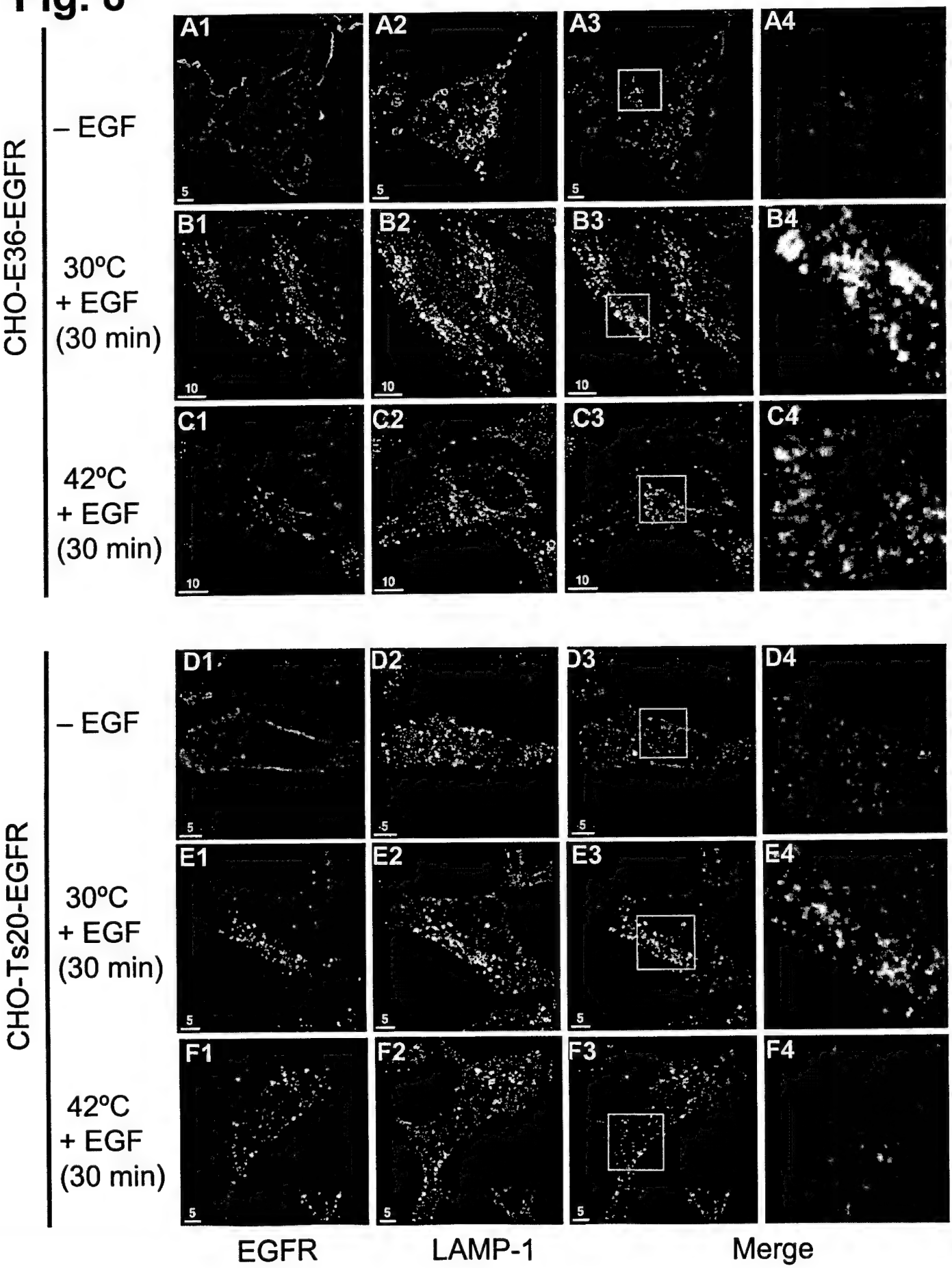

CHO-E36-EGFR 42°C
– EGF
+ EGF (5 min)
+ EGF (30 min)
CHO-Ts20-EGFR 42°C
– EGF
+ EGF (5 min)
+ EGF (30 min)
A1
A 2
A3
A4
B1
B2
B3
B4
C1
C2
C3
C4
D1
D2
D3
D4
E1
E2
E3
E4
F1
F2
F3
F4
EGFR
Transferrin
Merge

Fig. 8


CHO-E36-EGFR
– EGF
30ºC
+ EGF
(30 min)
42ºC
+ EGF
(30 min)
A1
A2
A3
A4
B1
B2
B3
B4
C1
C2
C3
C4
CHO-Ts20-EGFR
– EGF
30ºC
+ EGF
(30 min)
42ºC
+ EGF
(30 min)
D1
D2
D3
D4
E1
E2
E3
E4
F1
F2
F3
F4
EGFR
LAMP-1
Merge

Running title: Cbl mediates Vav ubiquitinylation.

Cbl-mediated ubiquitinylation and negative regulation of Vav

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Running title: Cbl mediates Vav ubiquitinylation.

ABSTRACT

The Cbl ubiquitin ligase has emerged as a negative regulator of receptor and non-receptor tyrosine kinases. Cbl is known to associate with Vav, the hematopoitetic-restricted Rac guanine nucleotide exchange factor proto-oncogene protein, but the consequences of this interaction remain to be elucidated. Using immortalized T cell lines from $Cbl^{+/+}$ and $Cbl^{-/-}$ mice, and transfection analyses in 293T cells, we demonstrate that Vav undergoes Cbl-dependent ubiquitinylation under conditions that promote Cbl and Vav phosphorylation. Interaction with Cbl also induced the loss of phosphorylated Vav. In addition, we show that an activated Vav mutant (Vav-Y174F) was more sensitive to Cbl-dependent ubiquitinylation. We demonstrate that the Cbl-dependent ubiquitinylation of Vav requires Cbl/Vav association through phosphorylated Y700 on Cbl, and also requires an intact Cbl RING finger domain. Finally, using transfection analyses in the Jurkat T cell line, we show that Cbl, but not its ubiquitin ligase mutant, can inhibit Vav-dependent signaling. Thus, our findings strongly support the role of Cbl, via its ubiquitin ligase activity, as a negative regulator of activated Vav.

Running title: Cbl mediates Vav ubiquitinylation.

INTRODUCTION

Activation of protein tyrosine kinases (PTKs) represents a fundamental mechanism by which higher eukaryotic cells respond to changes in the extracellular milieu. Less is known, however, about the mechanisms that fine tune the level of activation of PTKs and their downstream signaling pathways; such regulatory controls are essential to prevent pathological manifestations of PTK activation, such as cellular transformation or autoimmunity. Recent studies by us and others have revealed that the proto-oncogene product Cbl and the related family members Cbl-b and Cbl-c function as critical negative regulators of PTKs (1-8).

The PTK regulatory function of Cbl involves its ability to function as a ubiquitin ligase (E3) and thus target activated PTKs for ubiquitinylation. The N-terminal region of Cbl, composed of a four-helical bundle, a calcium-binding EF hand motif and an incomplete SH2 domain (1), functions as a Tyrosine Kinase-Binding (TKB) domain by directly interacting with specific autophosphorylation sites in activated PTKs, such as growth factor receptor tyrosine kinases (RTKs) and ZAP70/Syk PTKs (2-5). Alternatively, the proline-rich region in the middle part of Cbl protein can mediate its direct interaction with SH3 domains of Src-family kinases (6). The RING finger (RF) domain of Cbl, located between the TKB domain and the proline-rich region, binds to ubiquitin conjugation enzymes (Ubcs), such as UbcH5B and UbcH7 (7-11), providing a basis for the ubiquitin ligase activity of Cbl towards associated PTKs. The ubiquitin moiety attached to activated RTKs functions as a lysosomal sorting signal, resulting in

enhanced lysosomal degradation (12-15). Ubiquitinylation of Syk and Src-family kinases appears to target them for enhanced proteasomal degradation (14,16-19). Thus, Cbl-mediated ubiquitinylation provides a mechanism to eliminate the activated pools of PTKs via protein degradation, complementing dephosphorylation and other regulatory processes.

Studies with mammalian Cbl-b and Cbl-c, as well as *C. elegans* and *Drosophila* Cbl homologues, have demonstrated the conservation of this mechanism (20,21). Reflecting a critical role of Cbl-family proteins as negative regulators of PTK signaling, Cbl-deficient mice exhibit hypercellularity of lymphoid organs and increased branching morphogenesis of the mammary gland, whereas the Cbl-b deficient mice demonstrate fulminant autoimmune disease either spontaneously or upon immunization with autoantigens (22-24). Importantly, Cbl/Cbl-b double knockout mice are embryonic lethal (25).

While the functional roles of the association of Cbl with PTKs have begun to be elucidated, the role of Cbl interactions with other components of PTK signaling has remained less clear. Cbl is a major substrate of phosphorylation by various PTKs with which it interacts (6,8), resulting in stimulation-induced association of Cbl with SH2 domain-containing signaling proteins. Three well-characterized tyrosine phosphorylation sites in the C-terminal part of Cbl mediate its association with Vav (Y700), the p85 subunit of phosphatidylinositol-3 kinase (Y731), and the adaptor proteins of the Crk family (Y774) (4,6,8,26-28). The functional consequences of these associations are of

great interest. Two fundamentally opposite roles, which are not necessarily mutually exclusive, have been suggested. One view is that phosphorylated Cbl serves as a scaffold to assemble signaling complexes, thus facilitating PI 3-kinase, Vav and Crk-dependent cellular responses, such as cell survival and migration (20). Given the recent appreciation of the evolutionarily conserved function of Cbl proteins as ubiquitin ligases, we and others have suggested that Cbl may also target non-kinase signaling proteins for ubiquitinylation, which in turn may either target these proteins for degradation or regulate their function independent of degradation (20,29). Notably, Cbl-b was shown to constitutively interact with the p85 subunit of PI 3-kinase and induce PI 3-kinase ubiquitinylation, although a change in protein levels was not observed, nor did proteasome inhibitors increase the levels of ubiquitin-tagged PI 3-kinase. In conjunction with the inhibitory effect of PI 3-kinase inhibitors on the hyperactive phenotype of Cbl-b$^{-/-}$ T cells, these findings led the authors to suggest that Cbl-b-dependent ubiquitinylation negatively regulates PI 3-kinase in a degradation-independent manner (30), although it remains possible that the inability of the investigators to detect changes in the levels of PI 3-kinase may simply reflect a small size of the Cbl-associated pool. Thus there is a clear need to further elucidate the biochemical and functional consequences of the association of Cbl with non-PTKs targets. In this study, we have focused on the role of the association of Cbl with Vav.

Vav is a hematopoietic-restricted member of a family (Vav, Vav2, and Vav3) of guanine nucleotide exchange factors (GEFs) for the Rho family of small GTPases, especially Rac and Rho (31-34). Vav proteins contain a calponin-homology (CH)

domain, an acidic (Ac) region, a plekstrin-homology (PH) domain, a Dbl-homology (DH) domain characteristic of all Rho family GEFs, a zinc finger (ZF) domain, and two SH3 domains flanking a single SH2 region (31). Vav-mediated Rac1 activation plays an important role in antigen receptor-induced cytoskeletal reorganization, and activation of stress-activated protein kinases and important transcription factors, such as the nuclear factor of activated T cells (NF-AT) and NF-κB (35-38). Several biochemical and genetic studies have established a critical role for Vav in the development of T cells and their activation though the T cell receptor. Notably, Vav-deficient mice show a lack of IL-2 gene transcription in response to TCR triggering and impaired positive selection of thymocytes (39-42). Among over forty known human Rho/Rac GEFs, the Vav family is distinct in that the activity of these proteins is directly modulated by tyrosine phosphorylation (35). Vav is tyrosine phosphorylated in response to stimulation of the T and B cell antigen receptors in a Syk/ZAP70- and Src-family PTK-dependent manner (43-46). While phosphorylation of several tyrosine residues regulates Vav activity that of Tyr174 in the Ac region is quite critical, as it relieves the DH domain from the autoinhibition imposed by its intramolecular association with the N-terminal region (47). Indeed, a Y->F mutation at Tyr174 converts Vav into an oncogene, and enhances Vav-mediated cytoskeletal reorganization as well as JNK and NF-AT activation (48,49).

A number of recent studies suggest a potential role for Cbl in regulating Vav. Vav was shown to interact with Cbl in both thymocytes and peripheral T cells upon stimulation through the TCR (27). Phosphopeptide competition experiments suggested that the Cbl/Vav association was mediated by the Vav SH2 domain binding to

phosphorylated Y700 on Cbl (27). Notably, T cells from Cbl-b deficient mice showed enhanced Vav phosphorylation and TCR clustering upon TCR stimulation (23,24,50), while Cbl-b deficiency restored the defective TCR clustering in $Vav^{-/-}$ T cells (24). However, studies directly establishing a role for Cbl family proteins in the regulation of Vav are lacking, and it is unknown whether the Cbl ubiquitin ligase activity is involved in such regulation. Here, we demonstrate that Cbl functions as a ubiquitin ligase towards Vav, and that this activity allows Cbl to negatively regulate Vav-mediated signaling.

MATERIALS AND METHODS

Cells

293T human embryonic kidney epithelial cells were maintained in Dulbecco's modified Eagle's medium supplemented with 20mM HEPES, 1mM sodium pyruvate, 1mM nonessential amino acids, 100units/ml penicillin, 100μg/ml streptomycin, 50μM 2-ME (Life technologies, Gaithersburg, Md.) and 10% fetal bovine serum (Hyclone Inc.). JMC-T cell line, an SV40 T Ag-expressing derivative of Jurkat-JMC, was maintained as described (51). Immortal T cell lines 230 and 206, derived respectively from $Cbl^{+/+}$ and $Cbl^{-/-}$ mice (52), were maintained in RPMI 1640 containing supplements as above for 293T cells.

Antibodies

The antibodies used were: 12CA5, mouse monoclonal (mAb) anti-influenza hemagglutinin (HA) epitope tag (53); 4G10, mAb anti-phosphotyrosine (pY) (54); B-2, mAb anti-Vav (Santa Cruz Biotechnology); 4D10, mAb anti-Syk (Santa Cruz Biotech.); M2, mAb anti-FLAG (Invitrogen Corp.); OKT3, mAb anti-CD3 (ATCC); CD28.2, mAb anti-human CD28 (Pharmingen); H-211, rabbit polyclonal antibody (pAb) anti-Vav (Santa Cruz Biotech.); C-15, pAb anti-Cbl (Santa Cruz Biotech.); UG9510, pAb anti-ubiquitin (Affiniti Research Products, Ltd; UK).

Expression plasmids

The pAlter-MAX constructs encoding HA-tagged Cbl proteins, human Syk-Y323F mutant, pSRαNeo-CD8ζ (CD8α extracellular and transmembrane domains fused to TCRζ-chain cytoplasmic region) have been described (2). The constructs encoding HA-Cbl-Y700F, F700Y mutants (26), the NF-AT *firefly* luciferase reporter plasmid (55) and the pcDNA3.1-FLAG-Ub plasmid (56) have also been previously described. The pEF-myc-Vav encoding a myc-tagged Vav has been previously described (57), and was used to generate the Vav-Y174F mutant using the Quickchange Mutagenesis system (Invitrogen).

Transient transfection and cell lysis

293T cells were transfected using the calcium phosphate method, as previously described (52). The cell lysates used for coimmunoprecipitation studies were prepared in Triton lysis buffer (1% Triton X-100 [Fluka], 50mM Tris [pH 7.5], 150mM sodium chloride), whereas those used to assess ubiquitinylation were prepared in the more stringent RIPA buffer (Triton lysis buffer supplemented with 0.1% SDS and 0.5% sodium deoxycholate); each lysis buffer also contained 1mM phenylmethylsulfonyl fluoride, 1mM sodium orthovavadate, 10mM sodium fluoride, and 1μg per ml each of leupeptin, pepstain, antipain, and chymostatin (Sigma). The cell lysate protein concentration was determined by the Bradford assay (Bio-Rad Laboratories, Hercules, Calif.), with bovine serum albumin as the standard.

Immunoprecipitation and immunoblotting

Immunoprecipitations (IPs) from the indicated amount of cell lysate protein were performed as described (52), using protein A-sepharose 4B beads (Amersham-Pharmacia Biotech) as an immunosorbent. The IPs or cell lysates were resolved on SDS-PAGE gels, transferred to polyvinylidine difluoride (PVDF) membranes (NEN Life Science Produces), and serially incubated with the indicated primary antibodies and horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies (Cappel-Oreganon Technika, Durham, NC). Enhanced chemiluminescence (ECL) signals were recorded using a light-sensitive film (NEN Life Science Produces). Densitometry was carried out on directly scanned images using ScionImage for Windows^{TM} software.

NF-AT luciferase reporter assay

JMC-T cells were transfected with NF-AT luciferase together with Cbl and/or Vav expression plasmids using electroporation, as previously described (55). 18 h post-transfection, the cells were seeded in quadruplicates (2 x 10^5 cells/well) for each stimulation condition, and stimulated for 6-8 h at 37°C with media alone, 0.25μg/ml anti-CD3 mAb, or 0.25μg/ml each of anti-CD3 plus anti-CD28 mAb. Luciferase assays were performed on equal aliquots of cell lysates according to the manufacturer's instructions (Promega). Where indicated, the cells were cotransfected with a *Renilla* luciferase reporter plasmid (pRL-TK; Promega). *Firefly* (NF-AT) and *Renilla* luciferase activities were measured using a Dual Luciferase assay Kit (Promega), and NF-AT luciferase activity in each sample was normalized to *Renilla* luciferase activity to control for any variability in transfection efficiency, and are presented as mean ± standard deviation of

quadruplicate samples. All reporter assays were repeated at least three times. For biochemical analysis, an aliquot of the same cells that were used for the luciferase assay was cultured for a total of 48 h prior to cell lysis.

Stimulation of mouse T cell lines

T cell lines 230 and 206 were stimulated by incubation in medium containing 0.2 mM sodium orthovanadate or pervanadate (27) at 37°C for the indicated times.

RESULTS

Cbl-dependent Vav ubiquitinylation in T cells

Given the previously described association of Vav with Cbl upon TCR stimulation (27), and the ubiquitin ligase activity of Cbl towards a number of PTKs (14,20,58), we wished to determine if Vav is a target of Cbl-dependent ubiquitinylation. For this purpose, we analyzed a matched pair of virally immortalized T cell lines derived from $Cbl^{+/+}$ (cell line 230) and $Cbl^{-/-}$ (cell line 206) mice, respectively; we and others have previously used these cell lines to demonstrate the Cbl-dependence of Fyn and Lck ubiquitinylation and degradation (17,52), and the inhibitory role of Cbl in the localization of Lck to lipid-rich microdomains (59). As these cell lines do not express the surface TCR (data not shown), we used sodium orthovanadate or sodium pervanadate treatment to induce the activation of these cells. Anti-Vav IPs of unstimulated or pervanadate-stimulated cells were subjected to immunoblotting with anti-ubiquitin antibody to detect if Vav was ubiquitinylated. The stimulation of the $Cbl^{+/+}$ cell line (230) resulted in the appearance of slower migrating species recognized by the anti-ubiquitin antibody (Fig. 1A, first panel, lanes 3 vs. 4); these signals were substantially enhanced when stimulation was carried out in the presence of the proteasome inhibitor MG132 (Fig. 1B, first panel). In contrast to the $Cbl^{+/+}$ cell line, Vav ubiquitinylation was barely detectable in the pervanadate treated $Cbl^{-/-}$ cell line (206) (Fig. 1A, first panel, lane 4 vs. 2); even after treatment in the presence of MG132, the ubiquitin signals were dramatically lower compared to those in $Cbl^{+/+}$ cell line (Fig. 1B, first panel, lanes 2 vs. 4). One

consequence of ubiquitinylation is to target the substrate proteins for degradation by the 26S proteasome (60,61). In support of such a possibility, the level of ubiquitinylated Vav signal increased when cells were incubated in the presence of MG132 (above). However, stimulation of $Cbl^{+/+}$ or $Cbl^{-/-}$ cell lines with sodium orthovanadate did not lead to any detectable decrease in the overall Vav protein levels (Fig. 1C, second panel). Notably, however, orthovanadate stimulation of $Cbl^{+/+}$ cell line led to a time-dependent decrease in the level of phosphorylated Vav protein, as shown by anti-pY immunoblotting of anti-Vav IPs; reduction in phospho-Vav levels was visible within 10 min and continued to 30 min (Fig. 1C, first panel, lanes 7-8). In contrast, little if any decrease in phospho-Vav levels was seen in the orthovanadate-treated $Cbl^{-/-}$ cell line (Fig. 1C, first panel, lanes3-4). These results strongly suggest that Vav undergoes ubiquitinylation upon cellular activation via PTKs with an accompanying loss of phosphorylated Vav protein, and that Cbl is an important determinant of Vav ubiquitinylation and the concomitant loss of phospho-Vav protein.

The requirement of the Cbl RING finger domain and Y700 for Cbl-dependent Vav ubiquitination

The current model of Cbl-dependent ubiquitinylation, based primarily on studies of PTKs, is that Cbl associates with its targets via a protein-protein interaction and juxtaposes the RING finger-associated Ubc (E2) to the target protein. Therefore, we wished to assess if the motifs critical for Cbl/Vav association are required for Vav ubiquitinylation and if an intact RING finger domain in Cbl is necessary. For this

purpose, we established a reconstitution system in 293T cells, utilizing myc-tagged Vav and HA-tagged Cbl. As Vav and Cbl association in T cells is thought to involve the phosphorylation of Y700 in Cbl, we first determined the extent of Vav/Cbl association in transfected 293T cells with or without cotransfection of the relevant PTK, Syk. Syk was selected due to the availability of its mutant, Syk-Y323F, that fails to bind to the Cbl TKB domain and is therefore not subject to Cbl-mediated negative regulation (2,16), which would otherwise complicate the interpretation of the results. When Cbl and Vav were cotransfected in the absence of Syk, only a low level of association was observed in coimmunoprecipitation analyses (Fig. 2A, top panel, lane1). Concurrent transfection of either wildtype (WT) Syk or the Syk-Y323F mutant led to the expected phosphorylation of Cbl and Vav (second panel, lane 2 and3), and substantially increased the level of Cbl/Vav association (top panel, lanes 2 and 3). Thus, we chose the Syk-Y323F mutant for further analyses of Cbl/Vav interactions.

To assess if Cbl can target Vav for ubiquitinylation in the 293T cell reconstitution system, we transfected 293T cells with myc-Vav with or without HA-Cbl and Syk-Y323F, in addition to FLAG-tagged ubiquitin (FLAG-Ub). Anti-FLAG immunoblotting of anti-Vav IPs was used to detect the ubiquitinylated Vav, which appears as higher molecular weight species reactive with anti-FLAG antibody. Relatively low ubiquitinylated Vav signals were observed in the absence of co-transfected Syk-Y323F either with or without Cbl (Fig. 2B, top panel, lanes 1 and 2). Cotransfection of Syk-Y323F without Cbl resulted in a slight enhancement of the Vav ubiquitin signal (lane 3); however, a dramatic increase in ubiquitinylated Vav was seen when both Cbl and Syk-

Y323F were cotransfected (lane 4). As in the $Cbl^{+/+}$ T cell line discussted above, the enhanced Vav ubiquitinylation was not accompanied by a detectable decrease in Vav protein levels (Fig. 2B, second panel). However, when the same membrane was reprobed with an anti-pY antibody, a reduced phospho-Vav signal was detected in cells cotransfected with Cbl (third panel, compare lane 3 vs. 4), again suggesting that ubiquitinylation may specifically target the active (phosphorylated) pool of Vav for degradation. Consistent with this possibility, treatment of transfected 293T cells with the proteasome inhibitor MG132 partially countered the Cbl-dependent reduction in phospho-Vav levels (Fig. 2C, third panel, compare lanes 2 and 4); concomitantly, MG132 treatment resulted in an increase in the level of ubiquitinylated Vav signals (top panel, compare lanes 1 vs. 3 and 2 vs. 4).

To confirm that the slower migrating ubiquitinylated species represented Vav, and not cotransfected Cbl or Syk, we first resorted to immunoblotting with the respective antibodies. While Cbl and Syk signals were not detectable, the slower migrating species could not be categorically identified as Vav, apparently reflecting the small pool of Vav that is ubiquitinylated (data not shown). We therefore used an immunodepletion strategy to confirm that the observed ubiquitinylated species represent Vav and not associated Cbl or Syk proteins. Two rounds of immunodepletion resulted in greatly reduced Cbl levels and essentially undetectable levels of Syk in cell lysates, compared to the mock-depleted lysates (Fig. 2D, bottom two panels). However, such immunodepletion had no effect on the levels of ubiquitinylated species detected in anti-Vav IPs, strongly suggesting that these represent ubiquitinylated Vav, and not ubiquitinylated Cbl or Syk. Overall, the

results presented above indicate that Cbl, in the presence of a relevant tyrosine kinase, can target Vav for ubiquitinylation and subsequent degradation of the ubiquitinylated Vav pool.

Previous studies by Penninger and colleagues showed that the Cbl/Vav association in T cells was activation-dependent, and *in vitro* competition with phosphopeptides suggested that the interaction was mediated by the Vav SH2 domain binding to a conserved $pY^{700}MTP$ motif in Cbl (27). However, this conclusion has not been formally demonstrated *in vivo*. Thus, we first utilized the 293T cell cotransfection system to conclusively define the mode of Cbl/Vav association. Anti-HA immunoblotting of anti-Vav IPs from lysates of 293T cells cotransfected with HA-Cbl and Vav showed that an intact TKB (using Cbl-G306E mutant) and RING finger (using Cbl-C3AHN mutant) domains were not required for Cbl-Vav association (Fig. 3A, top panel, lanes 3 and 4 vs. lane 2). Importantly, a truncated Cbl mutant with intact TKB and RING finger domains as well as the proline-rich region (Cbl-655) did not associate with Vav, indicating that the Vav-binding site(s) resided within the C-terminal region of Cbl where the major phosphorylation sites are located. Indeed, a Cbl mutant (Cbl-Y5F) (26) with mutations of three well established (Y700, Y731 and Y774) and two putative (Y674 and Y735) phosphorylation sites was unable to associate with Vav (lane 5). Consistent with previous *in vitro* studies implicating Cbl Y700 as a potential binding site for the Vav SH2 domain, we found that the Cbl-Y700F mutant was unable to associate with Vav (lane 6). Remarkably, the ability of the Cbl-Y5F mutant to associate with Vav was restored by adding back Y700 (Fig. 3C, top panel, lane 3 vs. 2). As a control, we

demonstrated that HA-Cbl was not coimmunoprecipitated with an irrelevant antibody (Figure 3B), and that comparable levels of various HA-Cbl proteins were expressed in transfected cells as determined by anti-HA immunoblotting of cell lysates (Figure 3 A-C, bottom panels). These results conclusively demonstrate that the Cbl-Vav association is mediated by Vav binding to phosphorylated Y700 on Cbl.

Using the ubiquitinylation assay in 293T cells (as described above), we found that the Y700F mutation markedly reduced the ability of Cbl to mediate Vav ubiquitinylation (Fig. 3D, lane 5 vs. 3). Importantly, an intact RING finger domain was required for Cbl to induce Vav ubiquitinylation (lane 4). Thus, Cbl-mediated ubiquitinylation of Vav is critically dependent on the structural motifs within Cbl that mediate its physical interactions with the target (Vav) and the E2 enzyme of the ubiquitin pathway.

Enhanced Cbl-dependent ubiquitinylation of the activated Vav mutant, Vav-Y174F

One obvious reason for the requirement of a cotransfected PTK (Syk-Y323F) for Vav ubiquitinylation (above) is to induce Cbl phosphorylation, which in turn mediates the Vav-Cbl association (27). However, the Cbl-dependent decrease in phospho-Vav levels (Fig. 1C and 2B) and increased phospho-Vav levels in both Cbl-b and Cbl/Cbl-b deficient T cells (23,25,50), suggest that phosphorylation-dependent activation may render Vav more susceptible to Cbl-dependent ubiquitinylation. Y174 in Vav mediates a crucial intramolecular interaction that inhibits Vav activity, and its phosphorylation enhances Vav activity by promoting an open conformation (47). Notably, the Y174F

mutation induces the open conformation of Vav, and renders it constitutively active and oncogenic (48,49). We therefore used the Vav-Y174F mutant to assess if the activated Vav protein is more susceptible to Cbl-dependent ubiquitinylation. Notably, the level of association of Cbl with the Vav-Y174F mutant was substantially higher compared to wildtype Vav when analyzed in the absence of cotransfected Syk-Y323F (Fig. 4A, lane 1 vs. 3); comparable levels of association with Cbl were observed when Syk-Y323F was cotransfected (Figure 4A, compare lane 2 and 4). Importantly, the level of Cbl-dependent ubiquitinylation of Vav-Y174F mutant was substantially higher compared to WT Vav, both in the absence (Figure 4B, top panel, compare lane 1 and 4) and in the presence of cotransfected Syk-Y323F (compare lane 3 and 6). These results further support the idea that Cbl may preferentially bind the activated form of Vav and target it for ubiquitinylation.

Cbl inhibits Vav-dependent NF-AT activation

Given our findings that Vav undergoes PTK activation and Cbl-dependent ubiquitinylation and that phospho-Vav is degraded under these conditions, we wished to ask if Cbl indeed might function as a negative regulator of Vav signaling. Among the various functional readouts of Vav-dependent signaling, the transcriptional activation of the IL-2 promoter provides a convenient and sensitive assay. To this end, we utilized a reporter incorporating the NF-AT-binding sites of the IL-2 promoter linked to firefly luciferase. The overexpression of Vav is known to increase the level of TCR-induced NF-AT-luciferase activity (31), allowing an analysis of Cbl effects in the contexts of Vav

function. Thus, we carried out transient transfection of Jurkat T cells with plasmids encoding Vav and various Cbl proteins, together with the NF-AT-luciferase reporter. Overexpression of Vav led to a substantial increase of basal as well as anti-CD3 or anti-CD3 plus anti-CD28 induced NF-AT-luciferase activity, compared with that in the vector-transfected control cells (Fig 5A). Cotransfection of wild type Cbl substantially reduced the Vav-induced increase in basal as well as anti-CD3 or anti-CD3 plus anti-CD28 induced NF-AT-luciferase activity. Importantly, the Cbl-C3AHN mutant, lacking the ubiquitin ligase activity, failed to inhibit the Vav-dependent NF-AT-luciferase activity. The expression levels of Vav and Cbl proteins were confirmed by immunoblotting (Fig. 5B). These results support the role of Cbl as a negative regulator of Vav function through a mechanism that requires the ubiquitin ligase activity of Cbl.

DISCUSSION

Members of the Vav family (Vav, Vav2 and Vav3) of Rac/Rho GEFs have emerged as crucial signaling elements of tyrosine kinase-coupled cell surface receptors. The hematopoietic-restricted protein, Vav, is crucial for lympho-hematopoietic development and activation, and mutant forms of Vav can mediate oncogenesis. Thus, a better understanding of the biochemical mechanisms of Vav regulation is of considerable biological interest. Prior studies have shown that T cell receptor stimulation induces the association of Vav with Cbl, which has recently emerged as a negative regulator of receptor and nonreceptor tyrosine kinases by virtue of its ubiquitin ligase activity (12,14,16-18,20). Here, we have elucidated the mechanism by which Cbl interacts with Vav. We demonstrated that Vav-Cbl association leads to ubiquitinylation and degradation of active Vav, and show that Cbl can function as a negative regulator of Vav-mediated cellular activation.

We used immortal $Cbl^{+/+}$ and $Cbl^{-/-}$ T cell lines to establish that Vav undergoes Cbl-dependent ubiquitinylation and degradation when cellular tyrosine kinase pathways are activated. Complementary analyses in transfected 293T cells confirmed the ability of Cbl to target Vav for ubiquitinylation. Furthermore, analyses in both systems demonstrated that Cbl-dependent ubiquitinylation of Vav required tyrosine phosphorylation (by orthovanadate or pervanadate treatment, or cotransfection of Syk-Y323F). One likely explanation for the requirement for tyrosine phosphorylation was the postulated mechanism of Cbl/Vav interaction, namely via the Vav SH2 domain binding

to pY700 of Cbl (27). We used a panel of Cbl mutants to directly demonstrate that phosphorylation of Cbl Y700 was crucial as well as the predominant mechanism for Vav/Cbl association (Fig. 3A, B). Importantly, abrogation of the Vav/Cbl association via Cbl pY700 mostly eliminated the Cbl-dependent Vav ubiquitinylation. The low level of residual Vav ubiquitinylation in the presence of the Y700F Cbl mutant (Fig 3C) may represent the existence of minor additional mechanisms of association, such as either the Vav SH3 domain binding to the Cbl proline-rich region or the Vav SH2 domain binding to other phosphorylation sites on Cbl. Thus, the phosphorylation dependence of the Cbl-Vav association provides one likely mechanism for the requirement of tyrosine kinase activation for Cbl-dependent Vav ubiquitinylation. Furthermore, our analyses of an activated mutant of Vav (Vav-Y174F) showed it to be more susceptible to Cbl-dependent ubiquitinylation. A key mechanism that regulates the physiological activation of Vav is phosphorylation of regulatory tyrosine residues, including Y174, which leads to removal of the autoinhibition induced by the N-terminal domain interacting with the DH domain. Thus, the more open structure of Vav may either promote a more effective Vav-Cbl association (see Fig. 4A, lanes 3) or expose target lysine residues on Vav for Cbl-dependent ubiquitinylation. Regardless of their relative contributions, these mechanisms would ensure that Cbl selectively targets the activated pool of Vav for ubiquitinylation, a theme reminiscent of Cbl-dependent ubiquitinylation of tyrosine kinases (12,16,17,58,62). A further parallel is provided by the requirement of an intact Cbl RING finger domain for Cbl-dependent ubiquitinylation of Vav. These findings favor a model of Cbl-dependent Vav regulation analogous to that of Cbl-dependent regulation of tyrosine kinases: an

activation-dependent association step juxtaposes Cbl with its target (Vav) leading to its ubiquitinylation followed by functional regulation mediated by the ubiquitin tag.

In both the $Cbl^{+/+}$ and the $Cbl^{-/-}$ T cell lines, as well as the 293T transfection system, proteasome inhibitor treatment led to the accumulation of ubiquitinylated Vav, indicating that Cbl-dependent ubiquitinylation targets Vav for proteasomal degradation. However, an overall decrease in the levels of Vav protein was not seen under conditions where Vav ubiquitinylation was clearly observed. Given that the Cbl/Vav association and ubiquitinylation required tyrosine kinase activation, and that Vav activity is regulated by tyrosine phoshorylation, we reasoned that only the activated pool may be subjected to Cbl-dependent ubiquitinylation and degradation. Consistent with this idea, Cbl-dependent reduction of phosphorylated Vav level was observed in the $Cbl^{+/+}$ but not the $Cbl^{-/-}$ T cell line, and in Cbl-transfected 293T cells. Thus, we favor the idea that Cbl-dependent ubiquitinylation of Vav targets its activated pool for degradation. Cbl-b was shown to induce the ubiquitinylation of another non-tyrosine kinase target, PI 3-kinase, which also associates with Cbl proteins via a phosphorylation site. However, the authors did not observe Cbl-dependent changes in the levels of PI 3-kinase subunits or an effect of proteasome inhibitors, leading to the suggestion that Cbl-b inhibits the PI 3-kinase activity independent of degradation, although specific degradation of a small pool of activated PI 3-kinase could have gone undetected. Alternatively, Cbl-dependent ubiquitinylation of different target proteins may indeed lead to different biological consequences, as further exemplified by the Cbl-dependent ubiquitinylation of receptor tyrosine kinases, which provides a tag for lysosomal targeting (10,12-14).

It has been previously established that Vav participates in the induction of NF-AT/AP1-mediated transcription, and this function requires its GEF activity (63,64). We therefore used an NF-AT-luciferase reporter assay in Jurkat T cells to demonstrate that Cbl functions as a negative regulator of Vav-dependent cellular activation. Importantly, Cbl-mediated inhibition of Vav-induced NFAT-luciferase reporter activity required an intact RING finger domain, strongly supporting the role of Cbl-mediated ubiquitinylation in functional regulation of Vav. Consistent with the role of Cbl as a negative regulator of Vav, recent studies have shown that unstimulated as well as anti-CD3 stimulated thymocytes from Cbl $^{-/-}$ mice have markedly higher levels of Rac-GTP compared to those in Cbl$^{+/+}$ thymocytes (65). Notably, activated Cbl-b$^{-/-}$ T cells also showed higher levels of phospho-Vav and increased TCR clustering, a phenomenon dependent on Rac activation (23,50). As PI-3 kinase products are important in promoting the activation of Vav, loss of PI 3-kinase function could indirectly reduce the overall level of activated Vav. Our studies, however, establish a direct mechanism for Cbl-induced negative regulation of Vav. It is likely that in a physiological context, both direct and indirect mechanisms cooperate to mediate a more effective and integrated regulatory effect. Given that two Cbl family members can potentially regulate Vav and that three Vav family members exist, further studies will be necessary to precisely elucidate the redundant versus specific functional roles of the interactions that take place between the Cbl and Vav families of proteins. While Cbl$^{-/-}$ mice have significant abnormalities in T cell development, Cbl-b$^{-/-}$ mice show normal T cell development but a profound hypersensitivity of peripheral T cells to TCR triggering (23). It is notable that Cbl mRNA expression in thymocytes is far

greater than that of Cbl-b, whereas this is not the case in peripheral T lymphocytes (8,25). Recent studies have demonstrated that two other proteins, SOCS1 and hSIAH2, can also negatively regulate Vav via ubiquitinylation (57,66). Whether Cbl functions independently of or together with these other ubiquitin ligases are important questions for future studies.

In conclusion, we present evidence that Cbl functions as a negative regulator of Vav by targeting activated Vav for ubiquitinylation and degradation.

Running title: Cbl mediates Vav ubiquitinylation.

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Running title: Cbl mediates Vav ubiquitinylation.

Abbreviations:

PTK, protein tyrosine kinase; IP, immunoprecipitate; IB, immunoblot; mAb, monoclonal antibody; pAb, rabbit polyclonal antibody; RTK, receptor tyrosine kinases; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; WT, wildtype; TKB, tyrosine kinase-binding.

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Running title: Cbl mediates Vav ubiquitinylation.

FIGURE LEGENDS

Figure 1. **Pervanadate stimulation induces Vav ubiquitinylation and loss of phospho-Vav in $Cbl^{+/+}$ but not in $Cbl^{-/-}$ T cells.** *A*. Immortal $Cbl^{-/-}$ (206) and $Cbl^{+/+}$ (230) T cell lines were either left untreated (0 min) or treated with 0.2mM sodium pervanadate for 20 min at 37°C, and then lysed in PIRA buffer. Anti-Vav immunoprecipitations (IPs) from 0.5mg aliquots of lysate proteins were immunoblotted with anti-ubiquitin (Ub) antibody (top panel), followed by anti-Vav antibody (second panel). *B*. The $Cbl^{-/-}$ and $Cbl^{+/+}$ cell lines were treated with DMSO control (-) or 50μM MG132 (MG132) for 3 h and then treated with 0.2mM sodium pervanadate for 20 min at 37°C prior to cell lysis in RIPA buffer. Anti-Vav IPs from 0.5mg aliquots of lysate proteins were immunoblotted with anti-Ub antibody (first panel), followed by anti-Vav antibody (second panel). *C*. The $Cbl^{-/-}$ and $Cbl^{+/+}$ cell lines were treated with 0.2mM sodium vanadate for the indicated times. Anti-Vav IPs from 200μg aliquots of RIPA lysate proteins were immunoblotted with anti-pY antibody (top panel), followed by anti-Vav antibody (bottom panel). The level of phosphorylated Vav (top panel) and total Vav protein (second panel) were quantified by densitometry and the values are presented as fraction of the values for 0 min treatment (value of 1).

Figure 2. **Syk-dependent Vav ubiquitinylation by Cbl in 293T cells.** *A*. 293T cells were transfected with 0.5μg of Vav, 2μg of HA-Cbl and 0.5μg of CD8ζ expression plasmids with or without 50ng of WT-Syk or Syk-Y323F plasmids. 48 h post-transfection, cells were washed once with phosphate-buffered saline and lysed in Triton

lysis buffer. Anti-Vav IPs from 1mg aliquots of lysate proteins were resolved by SDS-PAGE and immunoblotted with anti-HA antibody (top panel), followed by anti-pY antibody (second panel) and anti-Vav antibody (third panel). 25μg aliquots of cell lysates were similarly resolved and immunoblotted with anti-Syk antibody (fourth panel) and anti-HA antibody (bottom panel). *B.* 293T cells were transfected as in *A*, using Vav, CD8ζ, with (+) or without (-) HA-Cbl and/or Syk-Y323F, as indicated. Anti-Vav IPs from 1mg aliquots of RIPA lysate proteins were resolved by SDS-PAGE and immunoblotted with anti-FLAG antibody (top panel), followed by anti-Vav (second panel) and anti-pY (third panel) antibodies. 25μg aliquots of cell lysates were similarly resolved and immunoblotted with anti-HA antibody. *C.* 293T cells were transfected as in *A*, using Vav, CD8ζ, Syk-Y323F and FLAG-Ub with (+) or without (-) HA-Cbl. Cells were treated with 50μM MG132 (+) or DMSO control (-) for 4h prior to harvesting the cells. Anti-Vav IPs from 1mg aliquots of RIPA lysate proteins were immunoblotted with anti-FLAG antibody (first panel), followed by anti-Vav (second panel) and anti-pY (third panel) antibodies. *D.* 293T cells were transfected as in *A*, using Vav, CD8ζ and FLAG-Ub, with (+) or without (-) Syk-Y323F and/or HA-Cbl. The RIPA lysates were subjected to two serial anti-Cbl or anti-Syk IPs (immunodepletion), and then 1mg aliquots of the mock-depleted or immunodepleted lysates were subjected to anti-Vav IPs, resolved by SDS-PAGE and immunoblotted with anti-Flag (first panel), followed by anti-Vav (second panel) antibodies. 25μg aliquots of mock-depleted or immunodepleted cell lysates were directly immunoblotted with anti-Cbl (third panel) and anti-Syk (fourth panel) antibodies.

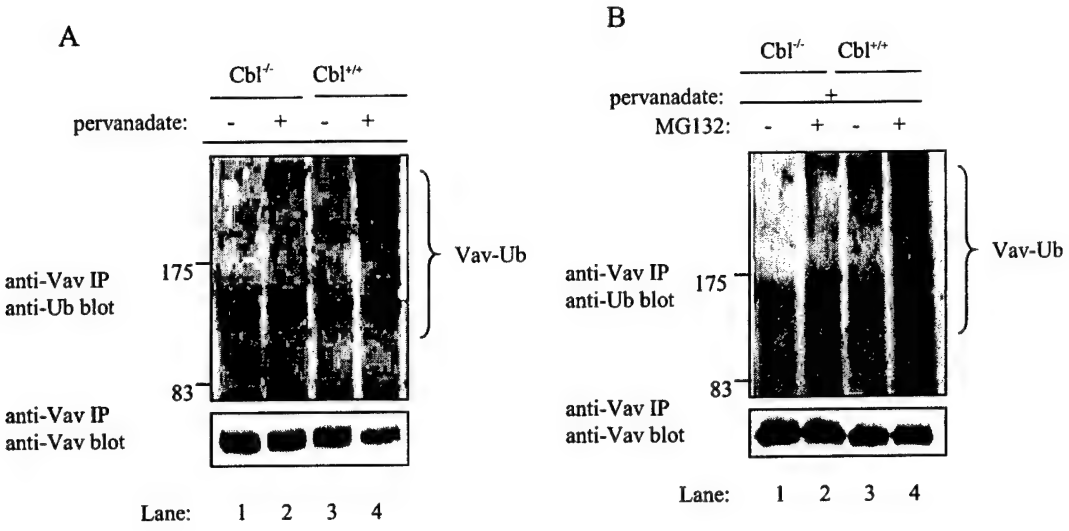
Figure 3. **The requirements for the Cbl/Vav association through Cbl Y700, and Cbl RING finger domain for ubiquitinylation of Vav.** *A*. 293T cells were transfected with 0.5μg of Vav, 2μg of wild type (WT) or mutant HA-Cbl construct and 0.5μg of CD8ζ, with or without 50ng of Syk-Y323F. 48 h post-transfection, cells were washed once with phosphate-buffered saline and lysed in Triton lysis buffer. Anti-Vav IPs from 1mg aliquots of lysate proteins were resolved by SDS-PAGE and immunoblotted with anti-HA (top panel) and anti-Vav (second panel) antibodies. 25μg aliquots of cell lysates were immunoblotted with anti-HA antibody (lower panel). *B*. 293T cells were transfected as in *A*, using HA-Cbl, CD8ζ, Vav and Syk-Y323F. Anti-Vav or isotype-matched control antibody IPs from 1mg aliquots of Triton lysate proteins were immunoblotted with anti-HA antibody (top panel), followed by anti-Vav antibody (second panel). 25μg aliquots of cell lysates were directly immunoblotted with anti-HA antibody (lower panel). *C*. 293T cells were transfected as in *A*, using WT or mutant HA-Cbl construct, Vav, CD8ζ and Syk-Y323F. Anti-Vav IPs from 1mg aliquots of RIPA lysate proteins were immunoblotted with anti-HA (first panel) and anti-Vav (second panel) antibodies. 25μg aliquots of cell lysates were directly immunoblotted with anti-HA antibody (lower panel). *D*. 293T cells were transfected as in *A*, using Vav, CD8ζ, Syk-Y323F and FLAG-Ub, with or without various HA-Cbl construct. Anti-Vav IPs from 1mg aliquots of RIPA lysate proteins were immunoblotted with anti-FLAG (first panel) and anti-Vav (second panel) antibodies. 25μg aliquots of cell lysates were immunoblotted with anti-HA antibody (lower panel).

Figure 4. **Enhanced Cbl-dependent ubiquitinylation of the activated Vav mutant, Vav-Y174F.** *A*. 293T cells were transfected with 0.5μg of WT Vav or Vav-Y174F and 2μg of HA-Cbl, CD8ζ, with (+) or without (-) 50ng of Syk-Y323F, and cell lysates were prepared 48h post-transfection. Anti-Vav IPs from 1mg aliquots of Triton lysate proteins were immunoblotted with anti-HA (top panel) and anti-Vav (second panel) antibodies. 25μg aliquots of cell lysate proteins were directly immunoblotted with anti-HA antibody (lower panel). *B*. 293T cells were transfected as in *A*, using WT Vav or Vav-Y174F, CD8ζ and FLAG-Ub, with (+) or without (-) HA-Cbl and/or Syk-Y323F. Anti-Vav IPs from 1mg aliquots of RIPA lysate proteins were immunoblotted with anti-FLAG (top panel) and anti-Vav (second panel) antibodies. 25μg aliquots of cell lysates were directly immunoblotted with anti-HA antibody (lower panel).

Figure 5. **Inhibition of Vav-dependent transactivation of NF-AT-luciferase reporter by Cbl, and the requirement of the Cbl RING finger domain.** *A*. Jurkat-derived JMC-T cells were transfected with plasmids encoding the NF-AT-luciferase reporter (10ì g), Vav (10ì g) and either WT or the RING finger mutant (C3AHN) of HA-Cbl (10ì g), together with pRL-TK plasmid (10ng) encoding *Renilla* luciferase as a transfection efficiency control. 18 h post-transfection, cells were plated in replicates of four and either left unstimulated or stimulated with anti-CD3 antibody with or without anti-CD28 antibody. NF-AT *(firefly)* luciferase activity in each sample was normalized to *Renilla* luciferase activity, and the data are presented as mean ± standard deviation of quadruplicates. All reporter assays were repeated at least three times, with similar results. *B*. Aliquots of the cell lysates used for luciferase assay were harvested after 48 h and

100μg aliquots of lysate proteins were used for immunoblotted with anti-HA and anti-Vav antibodies.

Fig 1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Cbl+/+
pervanadate:
- + - +
anti-Vav IP
anti-Ub blot
175
83
Vav-Ub
anti-Vav IP
anti-Vav blot
Lane: 1 2 3 4
B
Cbl-/-
Cbl+/+
pervanadate:
+
MG132:
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Vav-Ub
anti-Vav IP
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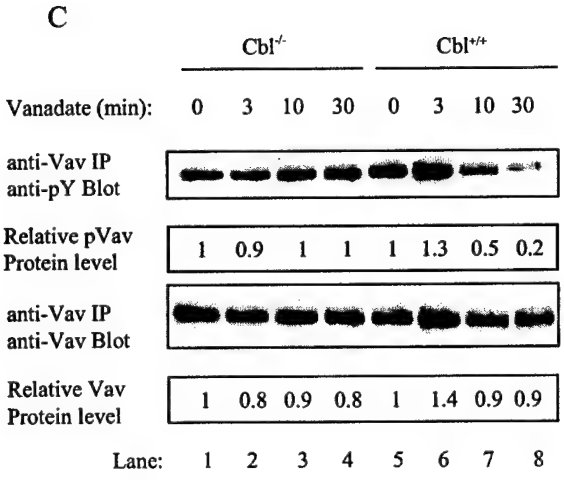
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Cbl+/+
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anti-pY Blot
Relative pVav
Protein level
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anti-Vav IP
anti-Vav Blot
Relative Vav
Protein level
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Fig.2

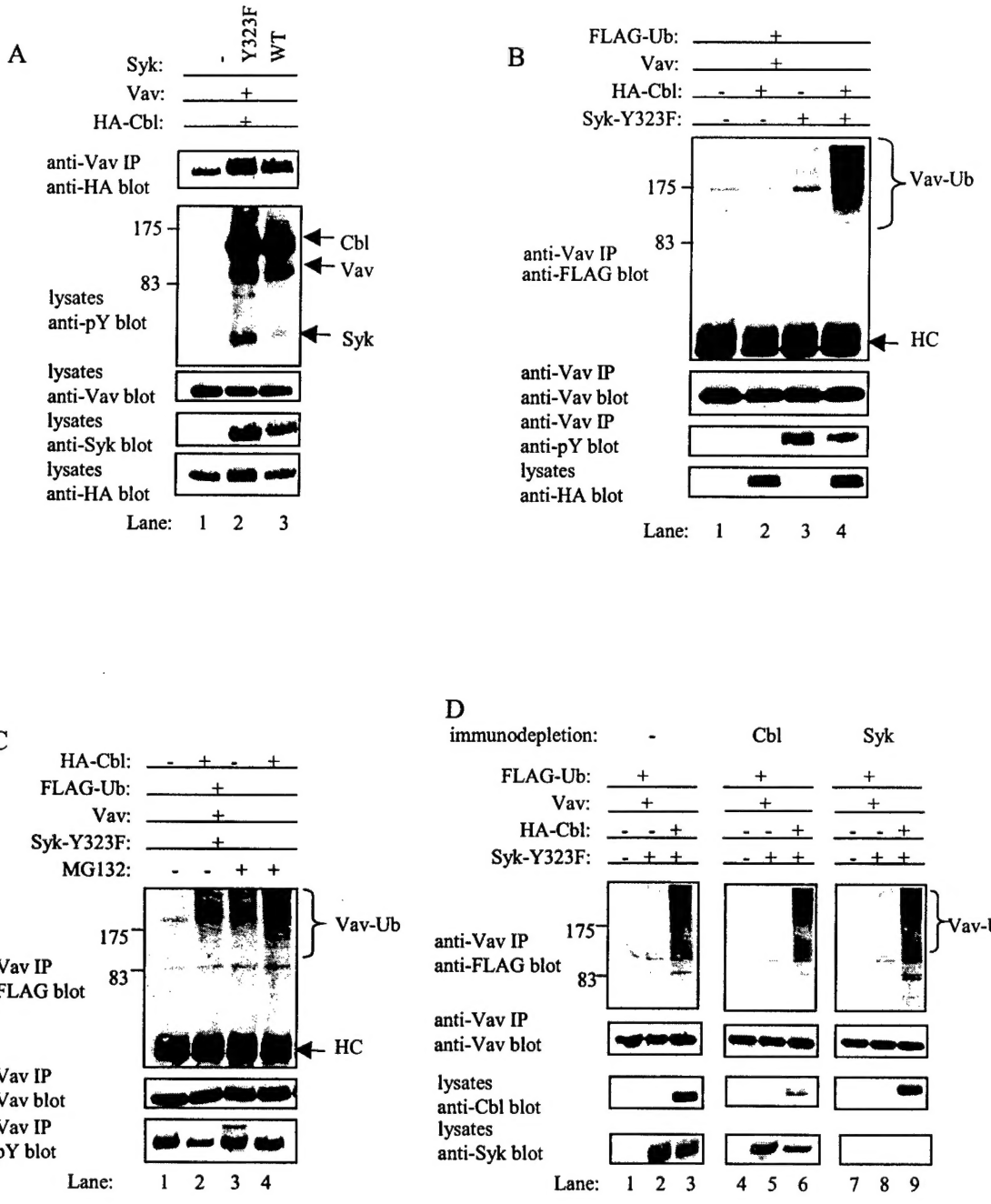
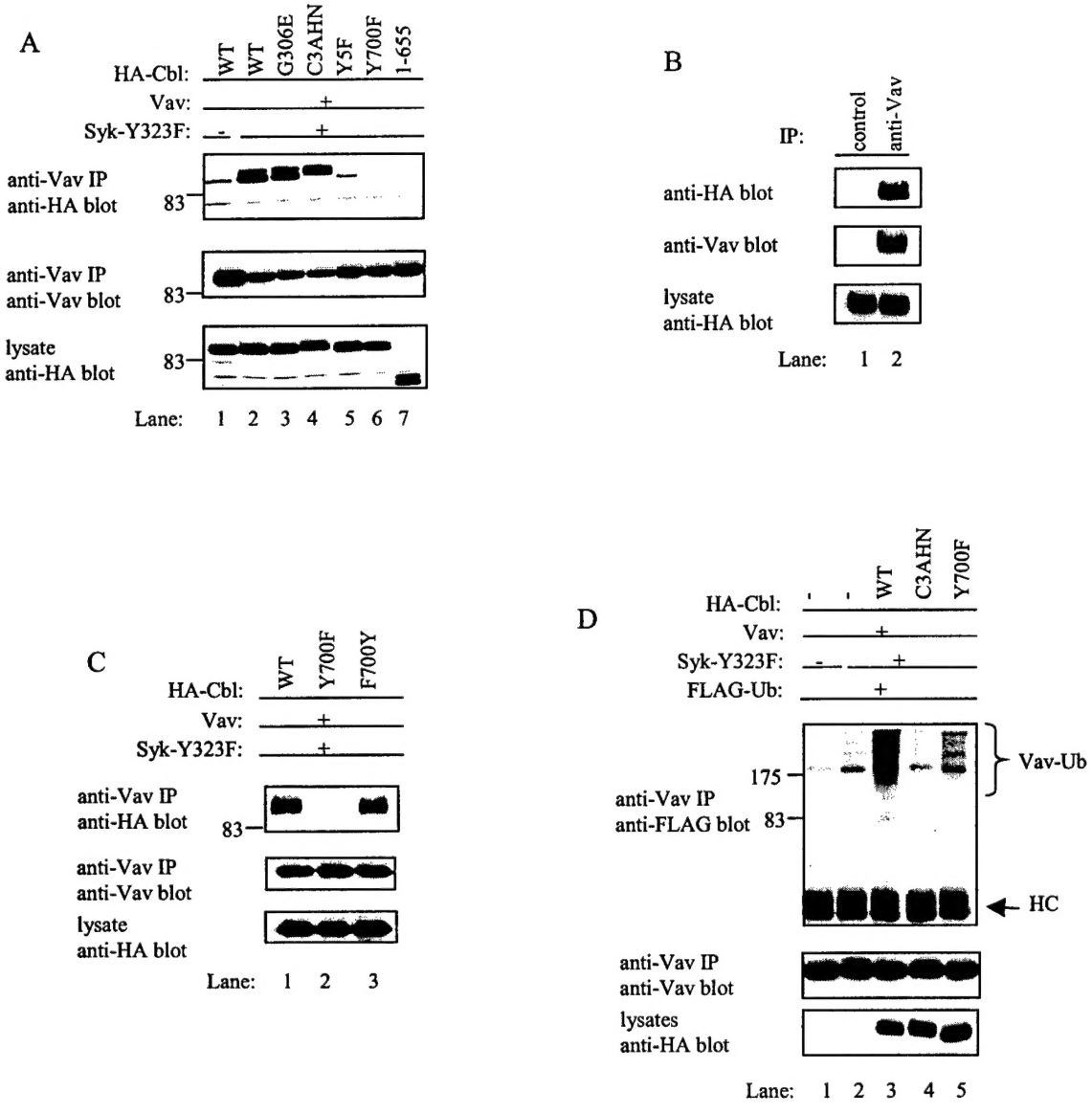

A
Syk: - Y323F WT
Vav: +
HA-Cbl: +
anti-Vav IP
anti-HA blot
175
83
Cbl
Vav
lysates
anti-pY blot
Syk
lysates
anti-Vav blot
lysates
anti-Syk blot
lysates
anti-HA blot
Lane: 1 2 3
B
FLAG-Ub: +
Vav: +
HA-Cbl: - + - +
Syk-Y323F: - - + +
175
83
Vav-Ub
anti-Vav IP
anti-FLAG blot
HC
anti-Vav IP
anti-Vav blot
anti-Vav IP
anti-pY blot
lysates
anti-HA blot
Lane: 1 2 3 4
C
HA-Cbl: - + - +
FLAG-Ub: +
Vav: +
Syk-Y323F: +
MG132: - - + +
175
83
Vav-Ub
anti-Vav IP
anti-FLAG blot
HC
anti-Vav IP
anti-Vav blot
anti-Vav IP
anti-pY blot
Lane: 1 2 3 4
D
immunodepletion: - Cbl Syk
FLAG-Ub: + + +
Vav: + + +
HA-Cbl: - - + - - + - - +
Syk-Y323F: - + + - + + - + +
175
83
Vav-Ub
anti-Vav IP
anti-FLAG blot
anti-Vav IP
anti-Vav blot
lysates
anti-Cbl blot
lysates
anti-Syk blot
Lane: 1 2 3 4 5 6 7 8 9

Fig.3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G306E
C3AHN
Y5F
Y700F
1-655
Vav:
Syk-Y323F:
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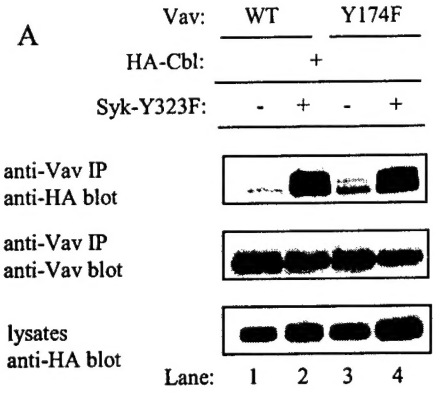
anti-HA blot
83
anti-Vav IP
anti-Vav blot
83
lysate
anti-HA blot
83
Lane: 1 2 3 4 5 6 7
B
IP:
control
anti-Vav
anti-HA blot
anti-Vav blot
lysate
anti-HA blot
Lane: 1 2
C
HA-Cbl:
WT
Y700F
F700Y
Vav:
Syk-Y323F:
anti-Vav IP
anti-HA blot
83
anti-Vav IP
anti-Vav blot
lysate
anti-HA blot
Lane: 1 2 3
D
HA-Cbl:
WT
C3AHN
Y700F
Vav:
Syk-Y323F:
FLAG-Ub:
175
anti-Vav IP
anti-FLAG blot
83
Vav-Ub
HC
anti-Vav IP
anti-Vav blot
lysates
anti-HA blot
Lane: 1 2 3 4 5

Fig.4


A
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HA-Cbl: +
Syk-Y323F: - + - +
anti-Vav IP
anti-HA blot
anti-Vav IP
anti-Vav blot
lysates
anti-HA blot
Lane: 1 2 3 4

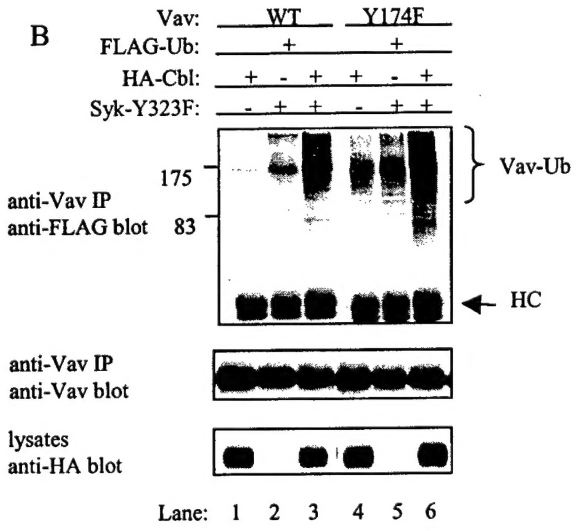
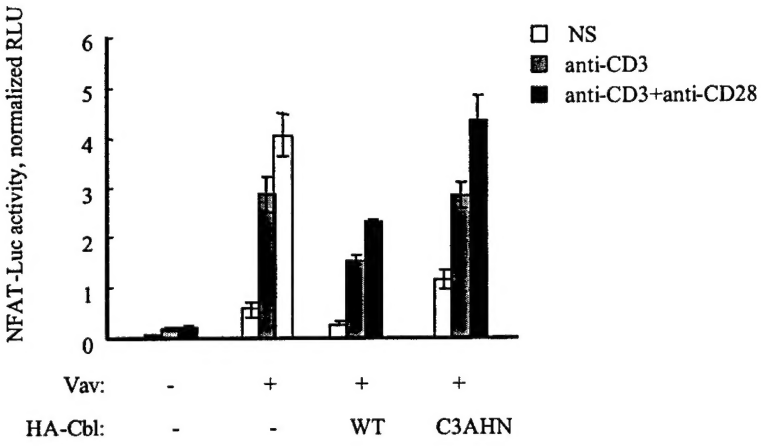

B
Vav: WT Y174F
FLAG-Ub: + +
HA-Cbl: + - + + - +
Syk-Y323F: - + + - + +
175
anti-Vav IP
anti-FLAG blot
83
Vav-Ub
HC
anti-Vav IP
anti-Vav blot
lysates
anti-HA blot
Lane: 1 2 3 4 5 6

Fig. 5

A


NFAT-Luc activity, normalized RLU
6
5
4
3
2
1
0
NS
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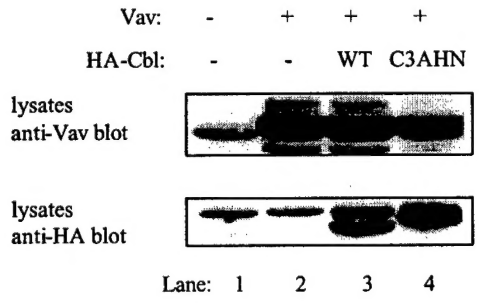
anti-CD3+anti-CD28
Vav: - + + +
HA-Cbl: - - WT C3AHN

B


Vav: - + + +
HA-Cbl: - - WT C3AHN
lysates
anti-Vav blot
lysates
anti-HA blot
Lane: 1 2 3 4